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Award Number: DAMD17-98-1-8033

TITLE: Regulation of Breast Carcinoma Chemotaxis by the Integrim Alpha6Beta4

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REPORT DATE: April 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2000	Annual Summary (16 Mar 99 - 15 Mar 00)

4. TITLE AND SUBTITLE
Regulation of Breast Carcinoma Chemotaxis by the Integrin Alpha6Beta4

5. FUNDING NUMBERS
DAMD17-98-1-8033

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report Contains Colored Photographs

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Breast carcinoma invasion is a complex process in which the normal balance of cellular adhesion, proteolysis and directed migration is altered leading to penetration of the basement membrane and underlying stroma. Work from our lab has shown that expression of the integrin α6β4 in breast carcinoma cells enhances their invasiveness. With funding from this grant, I have shown that integrin α6β4 expression in breast carcinoma cells leads to an increase in chemotactic (directed) migration toward lysophosphatidic acid (LPA) and is required for the lamellae formation. Both lamellae formation and chemotactic migration are inhibited or 'gated' by cAMP. My results reveal that a critical function of α6β4 is to suppress the intracellular cAMP concentration by increasing the activity of a rolipram-sensitive, cAMP-specific phosphodiesterase (PDE). Also, my results show that the small GTPase RhoA controls lamellipodial formation, is required for directed migration and is preferentially activated by the α6β4 integrin. Furthermore, the ability of α6β4 to influence cAMP metabolism is critical to Rho activation and function. I also find that cAMP metabolism and the α6β4 integrin can control the activation of Rac1, a protein that can counteract the functions of RhoA. The goal of this study is to understand how integrin α6β4 enhances lamellipodial formation and chemotaxis in breast carcinoma cells by identifying the signaling pathways involved in these processes.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer, integrin, cAMP, Rho, cytoskeleton	31
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction:

Tumor cell invasion and subsequent metastasis pose a serious threat to the survival of breast cancer patients. We currently need a better understanding of invasion in order to manage and eventually treat the late stages of breast cancer. The process of invasion requires a delicate balance between cellular adhesion, proteolysis and directed migration. The integrin α6β4, a receptor for laminin-1 (Lee, et al., 1992), laminin-2, laminin-4 (Spinardi, et al., 1995), and laminin-5 (Niessen, et al., 1994), has been linked to acquisition of an invasive phenotype and progression in multiple cancers (Falcioni, et al., 1994; Rabinovitz and Mercurio, 1996; VanWaes, et al., 1991). With breast cancer, recent studies have formed a strong link between integrin α6β4 and breast carcinoma invasion. First, Marchisio et al. have observed a striking localization of integrin α6β4 in invasive breast carcinoma. This observations support a previous study finding that expression of the integrin α6 subunit is more strongly associated with reduced patient survival than other markers including the estrogen receptor (Friedrichs, et al., 1995). Therefore, I believe that the integrin α6β4 plays a critical role in breast carcinoma invasion and progression and that the mechanism involved needs to be investigated.

Using an in vitro invasion assay system (Albini, et al., 1987), our group has shown that integrin α6β4, can enhance the invasive potential of MDA-MB-435 breast carcinoma (Shaw, et al., 1997) and the RKO colon carcinoma (Chao, et al., 1996) cell lines. Both the MDA-MB-435 and RKO cells express the laminin receptor integrin α6β1. Transfection of the integrin β4 subunit in these carcinoma cells results in the surface expression of the integrin α6β4 and increases the invasiveness of these cells. Furthermore, the expression of the integrin α6β4 does not lead to increased haptotactic migration on laminin for either the breast or colon carcinoma cell line. Since tumor cell invasion requires a delicate balance between cellular adhesion, proteolysis and directed migration, the integrin α6β4 signaling in this process likely involves the stimulation of one of these processes. I have recently resolved this issue by discovering that expression of the integrin α6β4 dramatically stimulates breast carcinoma cell chemotaxis, the directed migration toward a soluble attractant gradient, but not the proteolytic activity of these cells.

Currently, little is known about how integrin signaling impacts carcinoma cell chemotaxis and tumor progression. Ultimately, the results from this study should help to delineate the role of α6β4 integrin in facilitating chemotaxis and shed light on general signaling mechanisms that lead to a more invasive phenotype that characterizes late stage breast cancer. Toward this goal, I have made considerable progress during this second year of this fellowship.

Body:

The goal of this project is to identify signaling pathways involved in the invasion process that are enhanced by the α6β4 integrin. The α6β4 integrin was previously shown to promote carcinoma invasion by its activation of a phosphoinositide 3-OH (PI3-K) signaling pathway (Shaw et al., Cell 91:949-960). As described in my 1999 annual report, I demonstrate using MDA-MB-435 breast carcinoma cells that α6β4 stimulates chemotactic migration, a key component of invasion, but that it has no influence on haptotaxis. Stimulation of chemotaxis by α6β4 expression was observed in response to either lysophosphatidic acid (LPA) or fibroblast conditioned medium. Moreover, the LPA-dependent formation of lamellae in these cells is dependent upon α6β4 expression. Both lamellae formation and chemotactic migration are inhibited or 'gated' by cAMP and our results reveal that a critical function of α6β4 is to suppress the intracellular cAMP concentration by increasing the activity of a rolipram-sensitive, cAMP-specific phosphodiesterase (PDE). This PDE activity is essential for lamellae formation,

chemotactic migration and invasion based on data obtained with PDE inhibitors. Although PI3-K and cAMP-specific PDE activities are both required to promote lamellae formation and chemotactic migration, our data indicate that they are components of distinct signaling pathways. The essence of these findings is that α6β4 stimulates the chemotactic migration of carcinoma cells through its ability to influence key signaling events that underlie this critical component of carcinoma invasion. These data were published in the Journal of Cell Biology. This manuscript is appended to this report.

Of particular relevance to my work, Butcher and colleagues reported that cAMP is a negative regulator of leukocyte migration signaled through the classical chemoattractants (Laudanna, et al., 1997). In this model, cAMP impedes or gates RhoA-mediated leukocyte integrin activation and adhesion. Since LPA is a potent activator of RhoA (Moolenaar, et al., 1997), I wanted to explore the possibility that the integrin α6β4 releases cAMP gating of LPA-mediated RhoA activation that may lead to increased chemotaxis and lamellae formation. The Rho family of small GTPases, which includes Rho, Rac and cdc42, control the organization and remodeling of the actin cytoskeleton which is required for migration. These proteins have been implicated in the formation of stress fibers, lamellipodia and filopodia, respectively (Hall, 1998). Although much of our knowledge on their function has been obtained from studies with fibroblasts, other activities for these Rho GTPases have been observed recently in cells of epithelial origin. For example, Rac and cdc42 are required to maintain apical-basal polarity in epithelia (Jou and Nelson, 1998). Rho, interestingly, has been implicated in membrane ruffling in epithelial cells (Fukata, et al., 1999; Nishiyama, et al., 1994), a process attributed to Rac in fibroblasts (Hall, 1998). These findings are of particular interest with respect to our understanding of epithelial cell migration. The migration and invasion of epithelial-derived carcinoma cells are important phenomena that require the involvement of Rho GTPases (Itoh, et al., 1999; Keely, et al., 1997; Shaw, et al., 1997; Yoshioka, et al., 1998). For these reasons, we examined the hypothesis that RhoA is essential for the formation of actin-rich cell protrusions, the migration of carcinoma cells and, more importantly, that the activity of RhoA is regulated by the α6β4 integrin. In addition, we assessed the involvement of cAMP metabolism in these events.

For our initial studies, I used Clone A carcinoma cells which develop fan-shaped lamellae and exhibit random migration when plated on laminin-1, processes that are dependent on both the α6β4 and β1 integrins. To examine the hypothesis that RhoA functions in α6β4-dependent lamellae formation, clone A cells were co-transfected with a GFP construct and either a dominant negative RhoA (N19RhoA) or a control vector. Subsequently, the cells were plated onto laminin-1 and examined by phase-contrast microscopy. Clone A cells that expressed the control vector developed large lamellae with ruffled edges. In contrast, cells that expressed N19RhoA developed only a few small, fragmented lamellae that were devoid of membrane ruffles. Quantitative analysis of these images revealed that expression of N19RhoA reduced lamellar area by 80% in comparison to cells that expressed the control vector. Additionally, expression of N19RhoA inhibited the migration of clone A cells on laminin-1 by 70%. In contrast, expression of N17Rac did not inhibit the migration of clone A cells, although it did inhibit the migration of 3T3 cells by 85%. These data ,and the data described in the next four paragraphs, were published this past January in the Journal of Cell Biology. The manuscript is appended to this report.

Our observation that RhoA functions in lamellae formation and the migration of clone A cells, in conjunction with our previous finding that these events require the engagement of the α6β4 integrin (Rabinovitz and Mercurio, 1997), indicated that α6β4 may mediate the activation of RhoA. To assess RhoA activation, I used the Rho-binding domain of Rhotekin (RBD) to capture GTP-bound RhoA from cell extracts (Ren, et al., 1999). I find that the interaction of clone A cells with laminin-1, which requires α6β4, resulted in a significant amount of RhoA retained by RBD in comparison to the interaction of these cells with collagen I, which does not involve α6β4 directly. These experiments

were performed with cells that had been attached to laminin for 30 minutes because membrane ruffling was most apparent at this time. Quantitative analysis of the results obtained in four independent experiments revealed a three-fold greater increase in RhoA activation in cells plated on laminin-1 than in cells plated on collagen. To establish the ability of α6β4 to activate RhoA more definitively, we used integrin-specific mAbs to cluster both α6β4 and β1 integrins. I find that clustering of α6β4 resulted in an approximate two to three-fold higher level of RhoA activity in comparison to cells maintained in suspension.

The involvement of cAMP metabolism in migration, lamellae formation and α6β4-mediated RhoA activation was investigated using both IBMX, a phosphodiesterase (PDE) inhibitor, and H-89, a cAMP-dependent protein kinase (PKA) inhibitor. IBMX treatment, which prevents cAMP breakdown, inhibited the migration of clone A cells on laminin-1 almost completely. In contrast, inhibition of PKA with H-89 increased the rate of migration by four-fold. Together, these data indicate that cAMP inhibits or 'gates' carcinoma migration and lamellae formation, in agreement with our previous findings (O'Connor, et al., 1998). To establish the involvement of cAMP metabolism in the α6β4-mediated activation of RhoA, we used IBMX in the RBD assay. As shown in Fig. 3A, pretreatment of clone A cells with IBMX prior to plating on laminin-1 reduced the level of RhoA activation to that observed in cells plated on collagen. Importantly, IBMX did not inhibit either cell adhesion or spreading. Similar results were obtained with integrin clustering. These observations implicate cAMP metabolism in the α6β4-mediated activation of RhoA.

The data reported above raise the possibility that α6β4 influences RhoA localization because activation of RhoA is thought to involve its translocation to membranes (Bokoch, et al., 1994). To address this issue, clone A cells plated on either laminin-1 or collagen I were immunostained with a RhoA-specific Ab, as well as a β1-integrin specific Ab to mark membranes. In cells plated on collagen I, RhoA immunostaining was confined largely to the cytosol and it was distinct from the β1-integrin staining of the plasma membrane. In contrast, the α6β4-dependent interaction of clone A cells with laminin-1 resulted in the translocation of RhoA to membrane ruffles at the edges of lamellae where it co-localized with β1 integrin staining. However, RhoA did not co-localize with β1 integrins on the plasma membrane along the cell body. To assess the influence of cAMP metabolism on RhoA localization, clone A cells were pretreated with either IBMX or H-89 prior to plating on laminin-1. Inhibition of PDE activity with IBMX dramatically inhibited membrane ruffling and abolished RhoA localization in the few ruffles that persisted after IBMX treatment. Conversely, inhibition of cAMP-dependent PKA with H-89 resulted in an apparent increase in membrane ruffling and RhoA localization in membrane ruffles.

During the last year of this fellowship, I have made substantial progress in our understanding of the mechanism by which α6β4 functions in the dynamic processes of cell migration and lamellae formation by demonstrating that ligation of α6β4 with either antibody or laminin-1 results in the activation of RhoA and its translocation from the cytosol to membrane ruffles at the leading edges of migrating carcinoma cells. Importantly, we also provide evidence that the α6β4-mediated activation of RhoA is necessary for lamellae formation, membrane ruffling and migration. Furthermore, we establish that these events are regulated by cAMP metabolism and that they can occur independently of Rac1 involvement.

I also addressed the involvement of cAMP metabolism in the MDA-MB-435 breast carcinoma clones. In this system, the effects of cAMP metabolism is not as simple as in the clone A cells. We have previously shown that blocking cAMP phosphodiesterase activity with IBMX inhibits chemotaxis toward LPA in these cells (O'Connor, et al., 1998). Theoretically, blocking the downstream effector of cAMP, namely PKA, should reverse these effects. However this does not happen. As shown in Figure 1, the PKA inhibitor H-89 inhibited chemotaxis toward LPA in a dose-dependent manner. Furthermore, the effects of

IBMX and H-89 were additive, suggesting that cAMP signaling and its downregulation through phosphodiesterases are both important for migration.

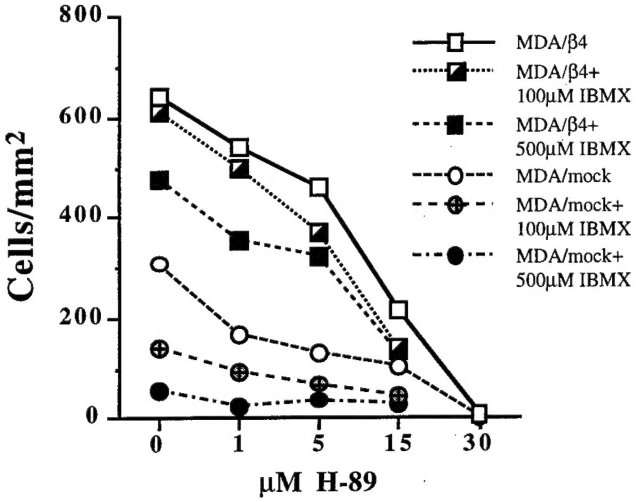

Figure 1. Inhibition of PKA by H-89 can not rescue the inhibitory effects of IBMX on MDA-MB-435 cell chemotaxis toward LPA. MDA-MB-435 cells which do not express α6β4 integrin (clone 6D7) or express wild type α6β4 integrin (clone 5B3) were treated with varying concentrations of H-89 or IBMX as noted for 30 minutes. The lower chamber of the Transwell dish was precoated with collagen I and then 50,000 cells were placed in the upper chamber. After 4 hours at 37°C, cells that did not migrate were removed from the upper chamber with a cotton swab and cells on the opposite side of the membrane were fixed, stained, and quantified manually as described previously (Shaw and Mercurio, 1994).

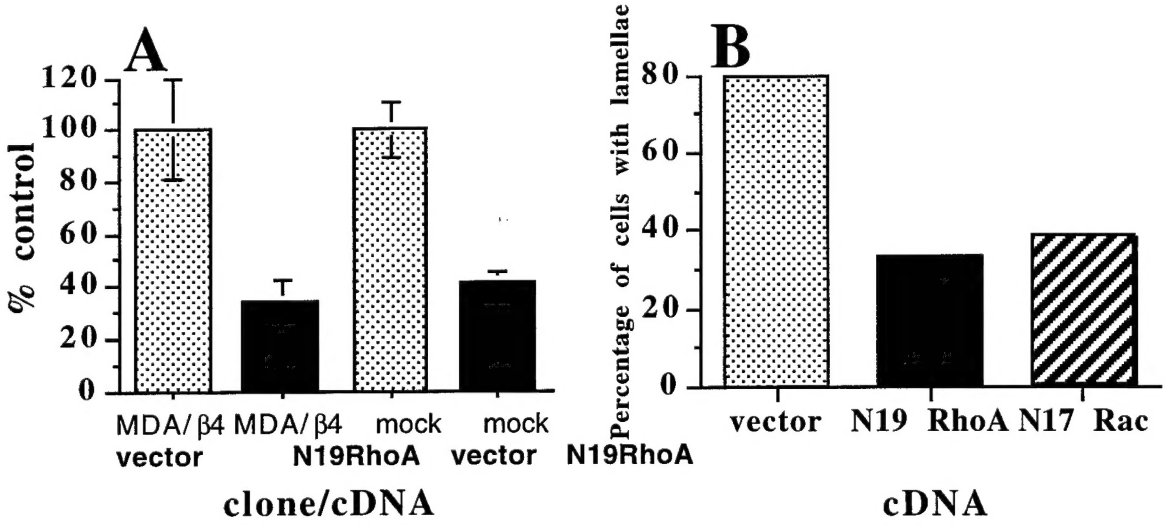

Figure 2. Dominant-negative constructs of RhoA or Rac inhibit chemotactic migration and lamellae formation in MDA-MB-435 cells. A) N19RhoA cDNA or vector alone were co-transfected with β-gal cDNA into MDA/β4 or MDA/mock cells. After 24 hours, cells were assayed for LPA-stimulated chemotaxis as described in Figure 1 and then stained for β-gal. Data are reported as number of β-gal staining cells migrated versus negative control (vector alone) ± standard deviation of triplicate determinations. B) MDA/β4 cells were co-transfected with either vector, N19RhoA or N17Rac cDNAs and a GFP reporter construct. After 48 hours, cells were plated onto LN-1 coated coverslips, treated with 100nM LPA for 5 minutes and then fixed. The percentage of GFP-positive cells that had lamellae were enumerated and are reported as the percentage of the total cell number from a representative experiment. Of note, N19RhoA and N17Rac expressing cells that formed lamellae had much smaller lamellae than control cells.

To address which Rho family GTPases were important for migration and lamellae formation in the MDA-MB-435 cells, I transiently transfected the MDA-MB-435 clones with a dominant negative construct of RhoA (N19 RhoA), Rac (N17Rac) or an empty vector. These transfectants were then assayed for their ability to chemotax or form lamellae in response to LPA. As shown in, expression of dominant negative RhoA (Figure 2A) and Rac (data not shown) in both the MDA/β4 and MDA/mock cells inhibited chemotactic migration compared to a vector only control. Interestingly, both constructs were also able to inhibit LPA stimulated lamellae formation in the MDA/β4 cells (Figure 2B). These data suggest that both RhoA and Rac are essential for chemotactic migration and lamellae formation in these cells.

Rac1 and cAMP are both known regulators of RhoA. I postulate that cAMP may regulate Rac1 by promoting its activation. I have preliminary evidence that this is indeed the case. I am currently exploring this avenue of investigation and am on track for this project as stated in my revised Statement of Works.

Conclusions:

During these two year of my fellowship, considerable progress has been made toward the understanding of the role of the α6β4 integrin in breast carcinoma invasion. The data that I have obtained demonstrate that the α6β4 integrin can stimulate lamellae formation and chemotactic migration of invasive carcinoma cells by increasing the activity of a rolipram-sensitive cAMP specific-PDE and lowering the $[cAMP]_i$. This cAMP specific-PDE functions in tandem with a PI3-K/Rac pathway, that is also regulated by α6β4 (Shaw, et al., 1997), and is required for carcinoma invasion and lamellae formation. This work resulted in a publication in the Journal of Cell Biology ((O'Connor, et al., 1998); publication appended to this report).

I have also obtained evidence that the target of cAMP-gating is RhoA, as has been observed previously in leukocytes. Using the Rhotekin binding assay to assess RhoA activation, we observed that engagement of α6β4 by either antibody-mediated clustering or laminin attachment resulted in a 2-3 fold increase in RhoA activation compared to cells maintained in suspension or plated on collagen. The α6β4-mediated interaction of clone A cells with laminin promoted the translocation of RhoA from the cytosol to membrane ruffles at the edges of lamellae and promoted its colocalization with β1 integrins, as assessed by immunofluorescence microscopy. In addition, RhoA translocation was blocked by inhibiting phosphodiesterase activity and enhanced by inhibiting the activity of cAMP-dependent protein kinase A. Together, these results establish a specific integrin-mediated pathway of RhoA activation that is regulated by cAMP and that functions in lamellae formation and migration. This work resulted in a publication in the Journal of Cell Biology ((O'Connor, et al., 2000); publication appended to this report). I am further investigating how cAMP may be necessary in cells that require Rac for migration and how α6β4 may control these signaling events.

In summary, the data that I have obtained supports my hypothesis that the integrin α6β4 amplifies signals required for lamellipodial formation that helps to promote chemotaxis. This fellowship has permitted me to investigate key signaling events that underlie carcinoma invasion and, I hope, will advance our understand and treatment of breast cancer.

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Key Research Accomplishments under this Fellowship:

- Expression of the integrin α6β4 stimulates the chemotactic migration of breast carcinoma cells in response to lysophosphatidic acid (LPA).
- Integrin α6β4 suppresses the intracellular cAMP concentration by increasing the activity of a rolipram-sensitive, cAMP specific phosphodiesterase (PDE4). This PDE4 activity is required for chemotactic migration and invasion of breast carcinoma cells.
- Lamellae formation in response to LPA in breast carcinoma cells is dependent upon expression of the integrin α6β4.
- The integrin α6β4 promotes the activation of RhoA, a protein vital for remodeling the actin cytoskeleton, lamellae formation and migration. This activation is dependent on PDE4 activity.
- Expression of α6β4 promotes the colocalization of RhoA with β1 integrins in membrane ruffles. This localization of RhoA is sensitive to cAMP metabolism.

Reportable outcomes:

Final Reports: The following are a list of publications and meeting abstracts that have resulted from the research efforts supported by this fellowship:

1. O'Connor, K. L., L.M. Shaw, and A. M. Mercurio. 1998. Release of cAMP gating by the α6β4 integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. J. Cell Biol. 143: 1749-1760.

2. O'Connor, K. L., L.M. Shaw , and A. M. Mercurio. 1998. Release of cAMP gating by the α6β4 integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. ASCB Annual Meeting Abstract.

3. O'Connor, K. L. and A. M. Mercurio. 1998. Integrin α6β4 Promotes Lamellae Formation and the Chemotaxis of Invasive Carcinoma Cells by Releasing cAMP Gating of RhoA Activation. The Function of Small GTPases: Keystone Symposium (meeting abstract)

4. O'Connor, K. L., Bao-Kim Nguyen, and A. M. Mercurio. 1998. RhoA Function in Lamellae Formation and Migration is Regulated by the α6β4 Integrin and cAMP Metabolism. J. Cell Biol. 148:253-258.

5. O'Connor, K. L. and A. M. Mercurio. 1999. RhoA Function in Lamellae Formation and Migration is Regulated by the α6β4 Integrin and cAMP Metabolism. Joint Regulation of Signaling Pathways by Integrins and Growth Factors: Keystone Symposium (meeting abstract)

There have been no patents or licenses, degrees, databases or animal models that have resulted from the funding of this fellowship.

I have not applied for further funding or employment based on the work suported by this grant.

As a result of the funding from this award, I had the opportunity to spend two weeks with Marco Conti at Stanford University. Dr. Conti is a recognized expert in the area of cAMP phosphodiesterases. Although I did not generate any reportable data from this trip, I learned a great deal from Dr. Conti and his lab. Furthermore, I have established a collaboration with him that I hope to pursue in the coming years that will enable me to help further our understanding of carcinoma cell migration.

Release of cAMP Gating by the α6β4 Integrin Stimulates Lamellae Formation and the Chemotactic Migration of Invasive Carcinoma Cells

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Abstract. The α6β4 integrin promotes carcinoma invasion by its activation of a phosphoinositide 3-OH (PI3-K) signaling pathway (Shaw, L.M., I. Rabinovitz, H.H.-F. Wang, A. Toker, and A.M. Mercurio. *Cell.* 91: 949–960). We demonstrate here using MDA-MB-435 breast carcinoma cells that α6β4 stimulates chemotactic migration, a key component of invasion, but that it has no influence on haptotaxis. Stimulation of chemotaxis by α6β4 expression was observed in response to either lysophosphatidic acid (LPA) or fibroblast conditioned medium. Moreover, the LPA-dependent formation of lamellae in these cells is dependent upon α6β4 expression. Both lamellae formation and chemotactic migration are inhibited or "gated" by cAMP and our results reveal that a critical function of α6β4 is to suppress the intracellular cAMP concentration by increasing the activity of a rolipram-sensitive, cAMP-specific phosphodiesterase (PDE). This PDE activity is essential for lamellae formation, chemotactic migration and invasion based on data obtained with PDE inhibitors. Although PI3-K and cAMP-specific PDE activities are both required to promote lamellae formation and chemotactic migration, our data indicate that they are components of distinct signaling pathways. The essence of our findings is that α6β4 stimulates the chemotactic migration of carcinoma cells through its ability to influence key signaling events that underlie this critical component of carcinoma invasion.

Key words: integrin • migration • cyclic AMP • phosphodiesterase • cytoskeleton

Carcinoma invasion is a complex process that involves directed migration and localized proteolysis (24). Although the mechanistic basis of invasion had been elusive, recent advances in molecular cell biology have facilitated a much more rigorous analysis of this important and critical component of cancer progression. In particular, insight into the function and regulation of cell adhesion receptors, as well as proteases, has fueled significant progress in our understanding of the invasive process. Studies aimed at defining specific signal transduction pathways that determine the behavior of invasive carcinoma cells are also contributing to an uncovering of the molecular basis of invasion.

Recent work by our group and others has implicated a key role for the α6β4 integrin in carcinoma invasion (3, 10, 32, 35, 40, 47). This integrin, which is a receptor for the laminins, is essential for the organization and maintenance of epithelial structure. In many epithelia, α6β4 mediates the formation of stable adhesive structures termed hemidesmosomes that link the intermediate filament cytoskeleton with the extracellular matrix (2, 12). The importance of this integrin in epithelial structure has been reinforced by the generation of β4-nullizygous mice that exhibit gross alterations in epithelial morphology and anchorage to the basement membrane (9, 46). In contrast to its function in normal epithelia, α6β4 can stimulate carcinoma migration and invasion through its ability to interact with the actin cytoskeleton and mediate the formation and stabilization of lamellae (32). This dynamic function of α6β4 in enhancing the migration of invasive carcinoma cells is quite distinct from its role in maintaining stable adhesive contacts in normal epithelia by associating with intermediate filaments. In fact, we have established that the ability of α6β4 to stimulate carcinoma migration and invasion depends upon its preferential activation of a phosphoinositide 3-OH kinase (PI3-K)[1]/Rac signaling pathway that we (40) and

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1. *Abbreviations used in this paper:* $[cAMP]_i$, intracellular cyclic AMP concentration; DIC, differential–interference contrast; Gi, inhibitory type G protein; IBMX, isobutylmethylxanthine; LPA, lysophosphatidic acid; PDE; phosphodiesterase; PI3-K, phosphoinositide 3-OH kinase.

others (18) have shown is necessary for invasion. In essence, our studies have defined an integrin-mediated mechanism of carcinoma invasion that involves the stimulation of carcinoma migration by the dynamic association of α6β4 with F-actin and the activation of a specific signaling pathway by this integrin.

Although we have established the involvement of α6β4 in the migration of invasive carcinoma cells, the nature of this migration has not been well defined. Moreover, signaling pathways distinct from PI3-K/Rac that are also regulated by α6β4 are likely to contribute to carcinoma migration. For these reasons, we sought to examine the migration mediated by α6β4 in more detail and to identify other signaling pathways regulated by this integrin that contribute to migration. The results obtained indicate that α6β4 stimulates the chemotactic migration of invasive carcinoma cells but that it has no influence on their haptotactic migration. Importantly, we demonstrate that the ability of α6β4 to suppress the intracellular cAMP concentration ($[cAMP]_i$) by activating a cAMP-specific phosphodiesterase (PDE) is essential for its enhancement of lamellae formation and chemotactic migration. Although PI3-K and cAMP-specific PDE activities are required for lamellae formation and chemotactic migration, we conclude that they are components of distinct signaling pathways.

Materials and Methods

Cell Culture and Antibodies

We used stable subclones of MDA-MB-435 human breast carcinoma cells that had been transfected with either the expression vector alone (mock transfectants), a full-length β4 cDNA (MDA/β4 transfectants), or a mutated β4 cDNA that lacked the entire cytoplasmic domain with the exception of four amino acids distal to the transmembrane sequence (MDA/β4-ΔCYT). The characterization of these transfectants has been described previously (38, 40). Both the β4 transfectants and the β4-ΔCYT transfectants expressed the α6β4 heterodimer on the cell surface as assessed by FACS® analysis and immunoprecipitation of surface-labeled extracts (40). The surface expression of α6β4 in these transfectants was comparable to the expression seen in other breast carcinoma cell lines that express this integrin endogenously such as MDA-MB-231 cells (Shaw, L.M., unpublished observation). All MDA-MB-435 cells were cultured in Dulbecco's modified Eagle's medium (DME) with 10% fetal calf serum plus 1% L-glutamine, 1% penicillin, and 1% streptomycin (GIBCO BRL, Gaithersburg, MD). Clone A cells, originally isolated from a human, poorly differentiated colon adenocarcinoma (7) and were cultured in RPMI 1640 medium containing 10% fetal calf serum plus 1% L-glutamine, 1% penicillin, and 1% streptomycin.

NIH-3T3 cells were cultured in DME containing 10% newborn calf serum plus 1% L-glutamine, 1% penicillin, and 1% streptomycin. NIH-3T3 conditioned medium was prepared from normal culture medium incubated with cells for 2 d before harvest with cellular debris removed by centrifugation.

The following function blocking, integrin-specific monoclonal antibodies (mAb) were used: mAb 13 (mouse anti-β1; S. Akiyama, National Institutes of Health, Research Triangle Park, NC), G0H3 (rat anti-α6; Immunotech, Westbrook, ME) and 2B7 (mouse anti-α6, prepared by our laboratory [39]). Non-specific mouse IgG was purchased from Sigma Chemical Co. (St. Louis, MO).

Migration and Invasion Assays

Cells were harvested using trypsin, rinsed three times with serum-free DME containing 250 μg/ml heat-inactivated BSA (DME/BSA), and then resuspended in DME/BSA. For migration assays, the lower surface of the membrane in each Transwell chamber (6.5-mm-diam, 8 μm pore size; Costar, Cambridge, MA) was coated for 30 min with either 15 μg/ml laminin-1 purified from Englebreth-Holm-Swarm tumor (19), 15 μg/ml collagen I (Vitrogen®; Collagen Biomaterials, Palo Alto, CA), or NIH-3T3 conditioned medium. For chemotaxis assays, either NIH-3T3 conditioned medium or lysophosphatidic acid (LPA) was added to the lower chamber. For haptotaxis assays, DME/BSA was added to the lower chamber. Cells (5×10^4) suspended in DME/BSA were added to the upper chamber. After incubating for 4 h at 37°C, nonmigrating cells were removed from the upper chamber with a cotton swab and cells that had migrated to the lower surface of the membrane were fixed with 100% methanol and stained with 0.2% (wt/vol) crystal violet in 2% ethanol. Migration was quantified by counting cells per square millimeter using bright-field optics. For antibody inhibition experiments, cells were incubated with 20 μg/ml of antibody for 30 min and then added to the Transwell chambers. The effects of pertussis toxin, IBMX (Calbiochem-Novabiochem, La Jolla, CA), forskolin, and rolipram (Sigma Chemical Co.) on migration were assessed by preincubating the cells with these reagents for 30 min before assay and including them in the assay medium at the concentrations noted in the figure legends.

Invasion assays were performed as described previously (40). In brief, 10 μg of Matrigel (Collaborative Research, Bedford, MA) was diluted with cold water and dried onto each Transwell filter. The Matrigel was reconstituted with DME for 1 h before its use in the assays. Cells were prepared as above and then added to the upper chamber of each well. NIH-3T3 conditioned medium was added to the lower chamber. Cells were allowed to invade for 4 h and then cells that had invaded were stained and quantified as described above.

cAMP Assays

Culture dishes (35-mm) were coated overnight with 20 μg/ml of collagen I in PBS and then blocked with serum-free RPMI containing 250 μg/ml BSA (RPMI/BSA). Cells (1.5×10^6) were then plated for 2 h and harvested by quickly removing the medium and extracting them directly with 80% (vol/vol) ethanol. Cell extracts were collected, cleared by centrifugation in a microcentrifuge for 10 min, dried in a SpeedVac (Savant Instruments, Farmingdale, NY) for 1.5 h, and then resuspended in 50 mM phosphate buffer, pH 6.2. The intracellular cAMP concentration was quantified using a cAMP enzyme-linked immunoabsorption assay (cAMP EIA; Cayman Biochemicals, Ann Arbor, MI) following the manufacturer's recommendation using nonacetylated cAMP as a standard and acetylcholine esterase-linked cAMP as a competitor. Values were corrected for cell number as determined from replicate plates. In some experiments, either 50 μM forskolin alone or forskolin plus 1 mM IBMX was added to cells 15 min before harvesting. To determine the cAMP content of cells under normal culture conditions, cells were plated in 35-mm dishes in DME plus 10% FCS, incubated for 18 h, and then processed as described above.

Phosphodiesterase Assays

cAMP PDE assays were performed according to the protocol of Sette et al. (37). In brief, cells were plated onto collagen I coated dishes as described for cAMP assays. Cells were then scraped from the dishes in a hypotonic lysis buffer (PDE lysis buffer: 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.2 mM EGTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 4 μg/ml aprotinin, and 2 mM PMSF) and then sonicated. Cellular debris was removed by centrifugation and the supernatants were assayed immediately for PDE activity. PDE activity of cell extracts (2–4 μg protein) was assayed in cAMP PDE assay buffer (20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1.25 mM β-mercaptoethanol, 0.14 mg/ml BSA, 1 μM cAMP, and 0.2 μCi [^{3}H] cAMP) for 10 min at 34°C. The reaction was stopped by adding 40 mM Tris, pH 7.5, containing 10 mM EDTA and heating for 2 min at 100°C. The reaction products were then digested with 50 μg *Crotalus atrox* snake venom (Sigma Chemical Co.) for 30 min at 34°C, separated from substrate using SpinZyme acidic alumina devices (Pierce Chemical Co., Rockford, IL), and quantified using a scintillation counter (Wallace, Gaithersburg, MD). Values were corrected for protein content and are reported as pmol cAMP hydrolyzed/min per milligram of protein. Protein content of cell extracts was determined using the Bio-Rad protein reagent (Hercules, CA) with BSA as a protein standard. Where noted, cells were incubated either for 30 min with 50 μM forskolin or 100 nM wortmannin or for 5 min with 100 nM LPA before harvest. For rolipram inhibition of PDE activity, 100 nM rolipram was added to cell extracts for 5 min before assaying for PDE activity.

Assessment of Lamellae Formation

Glass coverslips were coated overnight at 4°C with 20 μg/ml collagen I or laminin-1 and then blocked with BSA (0.25% in RPMI). The MDA-MB-435 transfectants were trypsinized and rinsed as described above, and then plated onto the coverslips for 2 h. As noted, cells were then treated with either 1 mM IBMX or 0.2% DMSO for 30 min. Subsequently, the cells were either treated with 100 nM LPA for 5 min or left untreated and then fixed for 10 min with 4% paraformaldehyde containing 10 mM Pipes, pH 6.8, 2 mM EGTA, 2 mM $MgCl_2$, 7% sucrose, and 100mM KCl. The coverslips were rinsed three times with PBS and mounted in glycerol. For the analysis of clone A cells, the cells were treated with IBMX or DMSO for 30 min, plated on laminin-1–coated coverslips, incubated for 45 min at 37°C, and then fixed. Clone A cells were then rinsed three times with PBS and incubated with blocking solution containing 1% BSA/5% normal donkey serum for 30 min. Cells were incubated with 20 μg/ml TRITC-labeled phalloidin in blocking solution for 30 min. Cells were rinsed four times with PBS over 30 min and then mounted in glycerol containing 1× PBS, pH 8.5, and 0.1% propylgallate. All cells were imaged with a Nikon Diaphot 300 inverted microscope (Tokyo, Japan) using either Nomarski differential–interference contrast (DIC) or phase–contrast optics. Images were captured with a charge-coupled device camera (Dage-MTI, Michigan City, IN), a frame grabber (Scion, Frederick, MD) and a 7600 Power Macintosh computer (Apple Computer, Cupertino, CA). Images were analyzed and lamellar area quantified using IPLab Spectrum image analysis software (Signal Analytics, Vienna, VA) using the criteria for defining lamellae used previously by our group (32). Lamellae were defined as broad, flat cellular protrusions rich in F-actin and devoid of membrane-bound vesicles. The lamellar area of each cell was determined using both phase contrast optics and FITC-phalloidin staining.

Analysis of PDE Expression

To determine the relative expression of PDE in the cells used in this study, cell extracts (40 μg of protein) were resolved by SDS-PAGE (8%), transferred to nitrocellulose, and then immunoblotted with PDE4-specific antibodies provided by M. Conti (Stanford University, Stanford, CA) (15). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and visualized using SuperSignal chemiluminescent substrate (Pierce Chemical Co.).

Analysis of PI3-K Activation

The activation of PI3-K by the integrin α6β4 was assessed as described previously (40). In brief, cells were trypsinized and rinsed as above, resuspended in RPMI/BSA at a concentration of 2×10^6 cells/ml and incubated for 30 min with integrin-specific antibodies or in buffer alone. Either IBMX (1 mM), forskolin (50 μM) or DMSO (0.2%) was added for 10 min before plating the cells onto tissue culture dishes coated with goat anti–rat IgG Ab. After incubation for 30 min at 37°C in the presence of IBMX, forskolin, or DMSO, the cells were washed twice with cold PBS and solubilized at 4°C for 10 min with 20 mM Tris, pH 7.4, 137 mM NaCl, 1% NP-40, 10% glycerol, 1 mM sodium orthovanadate, 2 mM PMSF, and 5 μg/ml of aprotinin, pepstatin, and leupeptin. Equivalent amounts of protein from each extract were incubated for 3 h at 4°C with the antiphosphotyrosine mAb, 4G10 (Upstate Biotechnology, Lake Placid, NY) and protein A–Sepharose (Pharmacia Biotech, Piscataway, NJ). The Sepharose beads were washed twice with lysis buffer then twice with 10 mM Hepes, pH 7.0, and 0.1 mM EGTA. Beads were then resuspended with kinase buffer plus 100 μM ATP, 25 μM $MgCl_2$, 10 μCi [γ-^{32}P]ATP, and 10 μl sonicated brain lipids and incubated for 10 min at room temperature. The reaction was stopped using 60 μl 2N HCl and 160 μl chloroform/methanol (1:1). Lipids were resolved using potassium oxalate-coated thin layer chromatography plates.

Results

Expression of the α6β4 Integrin in MDA-MB-435 Cells Enhances Their Chemotactic Migration

The possibility that expression of the α6β4 integrin influenced the rate of either haptotactic or chemotactic migration was assessed. For this purpose, stable transfectants of MDA-MB-435 cells were used that expressed either the α6β4 integrin (MDA/β4) or a deletion mutant of α6β4 (MDA/β4-ΔCYT) that retains only four amino acids of the β4 cytoplasmic domain, immediately proximal to the

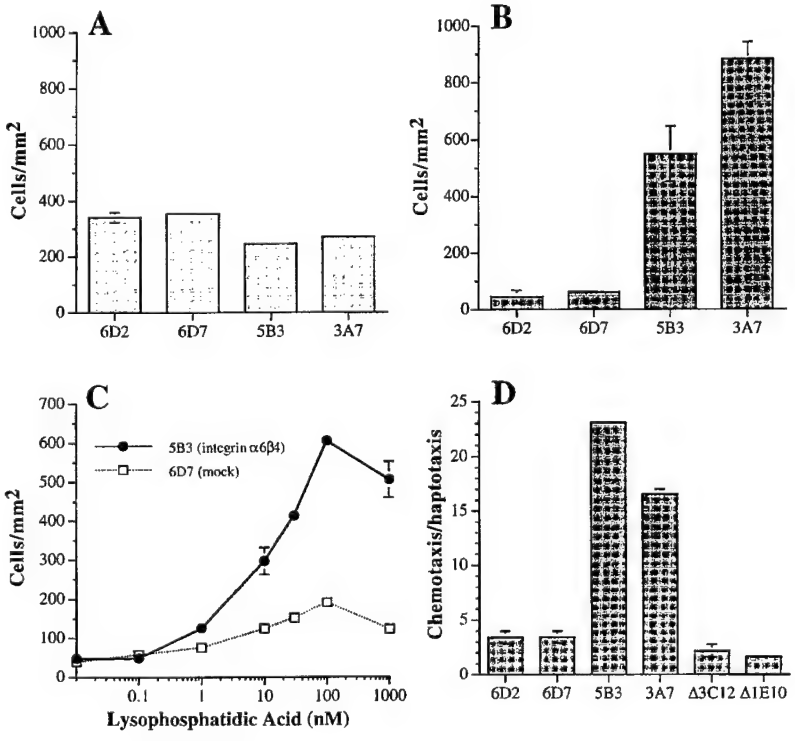

Figure 1. Expression of the α6β4 integrin in MDA-MB-435 carcinoma cells stimulates chemotaxis but not haptotaxis. The migration of the MDA/β4 (*5B3* and *3A7*) MDA/β4-ΔCYT (Δ3C12, Δ1E10), and MDA/mock (*6D2* and *6D7*) transfectants toward laminin-1 (haptotaxis; *A*), 3T3 conditioned medium (chemotaxis; *B*), or LPA (chemotaxis; *C* and *D*) was assessed using a modified Boyden chamber. The lower surfaces of Transwell membranes were coated with either laminin-1 (*A*), conditioned medium (*B*), or collagen I (*C* and *D*), and then either BSA (*A*) 3T3 conditioned medium (*B*) or LPA (*C* and *D*) was added to the lower chambers. Cells (10^5 [*A* and *B*] or 5×10^4 [*C* and *D*]) were placed in the upper chambers. After 4 h at 37°C, cells that did not migrate were removed from the upper chamber with a cotton swab and cells on the opposite side of the membrane were fixed, stained, and quantified manually as described in the Materials and Methods. (*A*) Haptotaxis toward laminin-1; (*B*) chemotaxis toward NIH-3T3 conditioned medium; (*C*) dose response of MDA-MB-435 subclones 5B3 (β4 transfected; *solid circles*) and 6D7 (mock transfected; *open squares*) chemotaxis toward LPA; (*D*) Chemotaxis toward 100 nM LPA. Data are reported as fold increases over haptotactic migration on collagen I in the absence of LPA. Data (all panels) are shown as mean ± standard deviation from triplicate determinations.

transmembrane domain (40). As shown in Fig. 1 *A*, subclones of the MDA/β4 transfectants (*5B3* and *3A7*) exhibited a rate of haptotactic migration toward laminin-1 that was slightly lower than the rate observed for subclones of the mock transfectants (*6D7* and *6D2*). In marked contrast, expression of α6β4 induced a substantial increase in the rate of chemotaxis of these cells towards conditioned medium from NIH-3T3 cells (Fig. 1 *B*). The rate of chemotaxis of the MDA/β4 transfectants (*5B3* and *3A7*) was 15–20-fold greater than that of the mock transfectants (*6D7* and *6D2*) over a 4-h time period. These data indicate that expression of α6β4 potentiates chemotactic migration of MDA-MB-435 cells without substantially altering their rate of haptotaxis.

To identify specific factors that could cooperate with α6β4 to promote chemotaxis of MDA-MB-435 cells, we tested several growth factors known to have chemotactic potential including epidermal growth factor, basic fibroblast growth factor, hepatocyte growth factor/scatter factor, insulin-like growth factor type I, transforming growth factor α and β, platelet-derived growth factor (AA and BB), somatostatin, thrombin, and LPA. Of these factors, only LPA was able to mimic the chemotactic effects of NIH-3T3 cell conditioned medium on the MDA-MB-435 transfectants (Fig. 1 *C* and data not shown). LPA stimulated the chemotaxis of MDA-MB-435 cells in a dose dependent manner with maximal stimulation observed at 100 nM. Of note, LPA stimulation of chemotaxis was five- to sevenfold greater in the MDA/β4 transfectants than in the mock transfectants. Subclones of the MDA/β4-ΔCYT transfectants (Δ3C12 and Δ1E10) exhibited a rate of chemotaxis that was similar to the mock transfectants (Fig. 1 *D*), indicating that the β4 cytoplasmic domain is critical for mediating the increased chemotaxis seen in the MDA/β4 transfectants.

The increased chemotaxis observed for the MDA/β4 transfectants in response to LPA was evident on both collagen I (Fig. 1, *C* and *D*) and laminin-1 (data not shown), indicating that α6β4-enhanced migration is independent of the matrix protein used for traction. This possibility was examined further by preincubating the MDA/β4 transfectants with function-blocking mAbs before their use in the chemotaxis assays. As shown in Fig. 2 *A*, inhibition of α6 integrin function with the mAb 2B7 did not block the chemotaxis of the MDA/β4 transfectants on collagen I towards LPA. However, this mAb inhibited the haptotaxis of MDA-MB-435 cells toward a laminin-1 gradient (Fig. 2 *B*), a process that is dependent on the α6β1 integrin (38). Chemotaxis toward LPA was inhibited completely, however, by preincubating the cells with the β1 integrin-specific mAb 13 (Fig. 2 *A*). Collectively, these data indicate that the stimulation of chemotaxis by expression of α6β4 can be independent of the adhesive functions of α6β4, and that the adhesive interactions required for α6β4-enhanced chemotaxis on collagen I are mediated through β1 integrins.

Expression of the α6β4 Integrin Is Required for the Formation of Lamellae in Response to LPA

Chemotactic migration frequently involves the formation of broad sheets of polymerized actin at the leading edge of the cell termed lamellae (27). To determine if expression of the α6β4 integrin influenced the formation of such motile structures, we analyzed the morphology of the MDA-MB-435 transfectants plated on collagen I (Fig. 3). Prominent lamellae were not evident in the mock transfectants and treatment with 100 nM LPA did not stimulate a significant increase in lamellar area (Fig. 3, *C* and *D*). The MDA/β4 transfectants exhibited a similar morphology to that of the mock transfectants when plated on collagen I (Fig. 3, compare *A* with *C*) or laminin-1 (data not shown). Within minutes after LPA treatment, however, the MDA/β4 transfectants formed large, ruffling lamellae (Fig. 3 *C*). Quantification of these cells by digital image analysis indicated that LPA stimulated a dramatic increase in the lamellar area of the two subclones of the MDA/β4 transfectants (Fig. 3 *D*). In contrast, no increase in the lamellar area of the mock transfectants in response to LPA was detected by this analysis (Fig. 3 *D*).

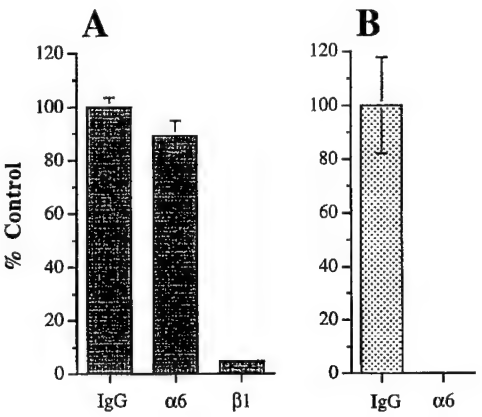

Figure 2. Inhibition of α6β4-stimulated migration by integrin-specific antibodies. MDA/β4 (5B3; *A*, *gray bars*) or mock transfectants (*6D7*; *B*, *stippled bars*) were incubated with the indicated function blocking mAbs for 30 min before their use in a chemotaxis assay using 100 nM LPA on collagen I (*A*) or a haptotaxis assay on laminin-1–coated wells (*B*) as described in Fig. 1. Nonspecific mouse IgG was used as a negative control. Data are reported as the percentage of migration observed for the IgG control ± standard deviation from triplicate determinations.

Pharmacological Evidence for the Involvement of cAMP in Chemotaxis

LPA is a bioactive phospholipid that can mediate its effects on cells through a receptor linked to heterotrimeric G proteins, including inhibitory type G (Gi) proteins (29). To assess the possible involvement of a Gi protein in α6β4-enhanced chemotaxis, we used pertussis toxin, which inactivates heterotrimeric Gi-proteins by ADP ribosylation (31). The LPA-stimulated chemotaxis of both the MDA/β4 and mock transfectants was inhibited by pertussis toxin with maximal inhibition observed at 100 ng/ml (data not shown). These data suggested that the α6β4 integrin enhances chemotaxis that is mediated through pertussis toxin-sensitive, Gi-linked receptors. Gi proteins are known to inhibit certain classes of adenyl cyclases and thus limit cAMP production (45). For this reason, we analyzed the impact of stimulating cAMP production on chemotaxis

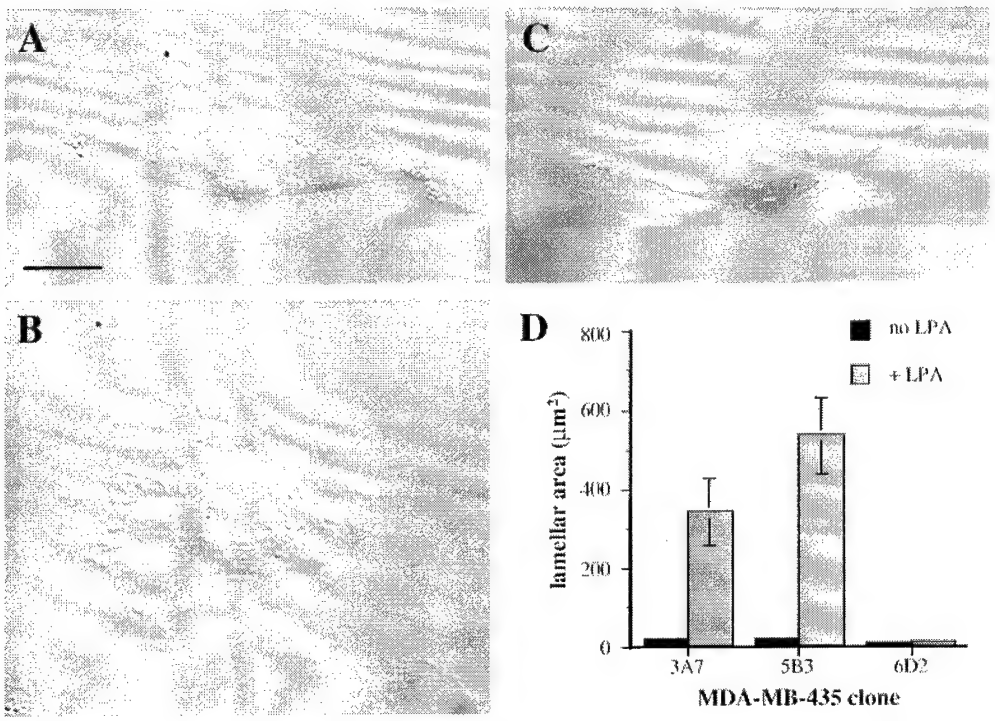

Figure 3. The α6β4 integrin is required for the LPA-dependent formation of lamellae in MDA-MB-435 cells. MDA/β4 (*A* and *B*) and mock transfectants (*C*) were plated onto coverslips that had been coated with 20 μg/ml collagen I. Cells were allowed to adhere for 2 h at 37°C and then treated with LPA for 5 min. (*B* and *C*) or left untreated (*A*). The cells were visualized using Nomarski DIC optics. Note the large lamellae that are formed in response to LPA stimulation of the MDA/β4 transfectants. (*D*) The effect of LPA on lamellar area was quantified using IPLab Spectrum imaging software. Data are shown as mean lamellar area ± standard error in which $n > 20$. Bar, 10 μm.

using forskolin. Although forskolin inhibited LPA-stimulated chemotaxis, the MDA/β4 and mock transfectants differed significantly in their response to this activator of adenyl cyclases. LPA-stimulated chemotaxis of the mock transfectants was inhibited to basal levels by 50 μM forskolin (Fig. 4 *A*). At this concentration of forskolin, the inhibition of chemotaxis of the MDA/β4 transfectants was only 50% and higher concentrations of forskolin (100 μM) did not abrogate chemotaxis of these cells (Fig. 4 *A*). Interestingly, treatment of the MDA/β4 or mock transfectants with forskolin did not inhibit haptotactic migration on laminin-1 (Fig. 4 *B*). These data indicate that a cAMP-sensitive pathway plays a key role in LPA-stimulated chemotaxis of MDA-MB-435 cells and they suggest that the α6β4 integrin may regulate this pathway.

Expression of the α6β4 Integrin in MDA-MB-435 Cells Influences cAMP Metabolism

To determine if α6β4 expression influences the $[cAMP]_i$, the $[cAMP]_i$ was determined in extracts obtained from subconfluent cultures of MDA/mock, β4, and β4-ΔCYT transfectants using a cAMP enzyme-linked immunoabsorption assay. As shown in Fig. 5 *A*, the MDA/β4 transfectants had a 30% lower $[cAMP]_i$ (2.7 pmol cAMP per 10^6 cells) than either the mock (3.7 pmol cAMP per 10^6 cells) or β4-ΔCYT transfectants (3.8 pmol cAMP per 10^6 cells). This difference was statistically significant ($P < 0.001$). Of note, neither clustering of α6β4 using the 2B7 mAb and an appropriate secondary Ab nor LPA treatment reduced cAMP levels further (data not shown).

The observation that the MDA/β4 transfectants were more resistant to forskolin inhibition of chemotaxis than the mock transfectants (Fig. 4) suggested that these two populations of cells differ in their ability to metabolize the cAMP generated in response to forskolin stimulation. This possibility was examined by determining the $[cAMP]_i$ in forskolin-treated cells. As shown in Fig. 5 *B*, the MDA/β4 transfectants exhibited a 30% lower $[cAMP]_i$ than the mock transfectants when plated on collagen I. With forskolin stimulation, a 2.5-fold greater accumulation of cAMP was observed in the mock transfectants (6.6 pmol per 10^6 cells) compared with the β4 transfectants (2.6 pmol per 10^6 cells). When the forskolin-treated cells were also treated with the PDE inhibitor, IBMX, to prevent breakdown of cAMP, the MDA/β4 transfectants exhibited a $[cAMP]_i$ comparable to the mock transfectants (120 ± 11 versus 104 ± 18 pmol per 10^6 cells, respectively; Fig. 5 *C*). Together, these data suggest that expression of α6β4 integrin suppresses the $[cAMP]_i$ by increasing PDE activity.

To establish more directly that expression of the α6β4 integrin can regulate cAMP-dependent PDE activity, the activity of this enzyme was assayed in cell extracts obtained from the MDA/mock and β4 transfectants. As shown in Fig. 6 *A*, the MDA/β4 transfectants exhibited a significantly higher rate of PDE activity than the mock transfected cells. Moreover, the PDE activity of the MDA/β4 transfectants was markedly increased (51% for 5B3 and 45% for 3A7) in response to forskolin stimulation compared with the mock transfectants (29% for 6D7; Fig. 6 *A*). The difference in PDE activity between the MDA/β4 and mock transfectants was eliminated by rolipram, a type IV PDE-specific (PDE 4) inhibitor (Fig. 6 *B*). These data indicate that a cAMP-dependent PDE 4 activity is influenced by α6β4 expression in MDA-MB-435 cells. Also, this activity is likely responsible for the observed decrease in $[cAMP]_i$ and the resistance to forskolin-mediated inhibition of LPA chemotaxis observed in the MDA/β4 transfectants.

To examine the possibility that the MDA/β4 and mock

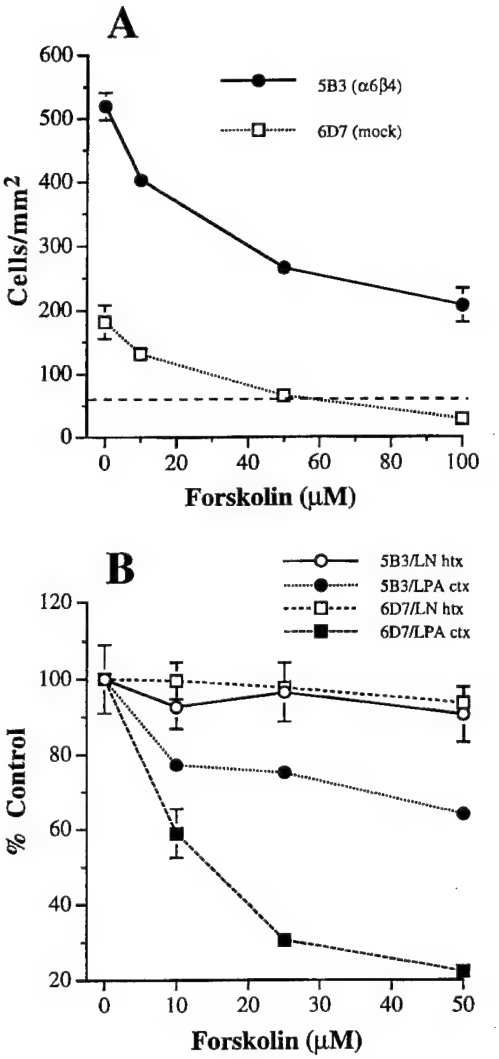

Figure 4. Forskolin stimulation of adenyl cyclase inhibits LPA-mediated chemotaxis differentially in the MDA/β4 and mock transfectants. (*A*) MDA/β4 transfectants (*5B3*; *solid circles*) or mock transfectants (*6D7*; *open squares*) were treated with the indicated concentration of forskolin for 30 min before their addition to the upper wells of the Transwell chambers. Cells were assayed for LPA-mediated chemotaxis on collagen I as described in Fig. 1. The dashed line depicts the basal level of migration of both subclones in the absence of LPA. (*B*) In a separate experiment, the same cells were treated with forskolin for 30 min before assaying for LPA chemotaxis (*solid symbols*) or laminin haptotaxis (*open symbols*). Data are reported as the percent migration of cells not treated with forskolin ± standard deviation of triplicate determinations.

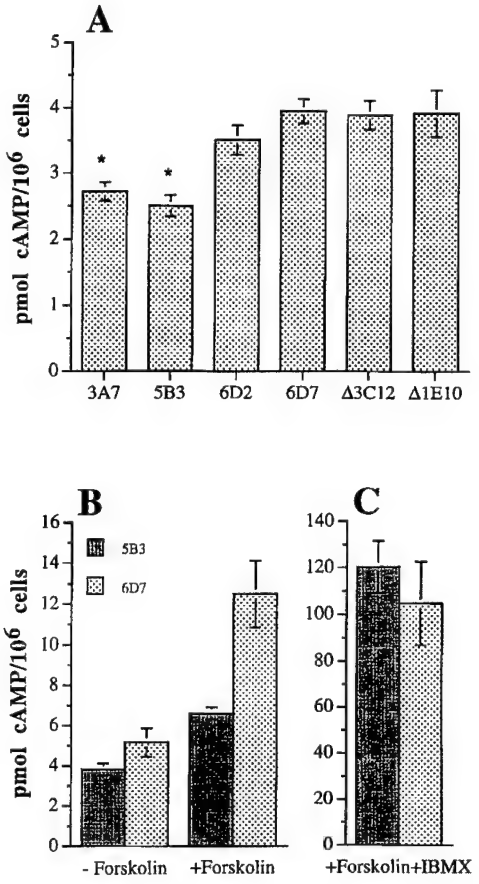

Figure 5. Intracellular cAMP content of the MDA-MB-435 transfectants. The MDA/β4 (*3A7*, *5B3*), MDA/β4-ΔCYT (*Δ3C12*, *Δ1E10*) and MDA/mock transfectants (*6D2*, *6D7*) were plated in DME containing 10% FCS. After 18 h, cells were harvested and cAMP content was measured using a cAMP EIA protocol as described in Materials and Methods. Data shown represent the mean of 10 sample determinations ± standard error. The difference in the $[cAMP]_i$ between the MDA/β4 and the mock transfectants is significant ($P < 0.001$; *asterisk*), but the difference between the mock and the β4-ΔCYT transfectants is not significant ($P = 0.2$). (*B* and *C*) Differential effects of forskolin stimulation on the $[cAMP]_i$ in the MDA/β4 and mock transfectants. The $[cAMP]_i$ was assayed in the 5B3 (*solid bars*) and 6D7 (*stippled bars*) clones plated on collagen I and treated for 15 min with either 50 μM forskolin (*B*) or forskolin and 1 mM IBMX (*C*). Note that the MDA/β4 transfectants (*5B3*) are more resistant to a forskolin-stimulated increase in $[cAMP]_i$ than the mock transfectants (*6D7*). The inhibition of PDE activity with IBMX shown in *C* reveals that α6β4 expression results in an increase in PDE activity and not a decrease in cAMP synthesis. Data shown are the mean values ± standard error obtained from multiple experiments.

transfectants differed in their level of PDE expression, we assessed PDE 4 expression in these cells using antibodies specific for the various PDE 4 variants (15). The predominant PDE 4 variant expressed in MDA-MB-435 cells is PDE 4B based on results obtained with antibodies specific for PDE 4A, 4B and 4D (data not shown). Importantly, the expression of PDE 4B did not differ significantly between the MDA/β4 and mock transfectants (Fig. 6 *C*). These data indicate that the increased PDE activity observed in the MDA/β4 transfectants is not the result of increased PDE expression.

PDE Activity Is Necessary for Chemotaxis, Invasion, and Lamellae Formation

The importance of PDE for chemotactic migration was examined by treating the MDA/mock and β4 transfectants with IBMX before their use in the chemotaxis assay. As shown in Fig. 7 *A*, IBMX inhibited LPA-stimulated chemotaxis with maximal inhibition observed at 1 mM. Similar results were obtained with the cAMP-specific PDE inhibi-

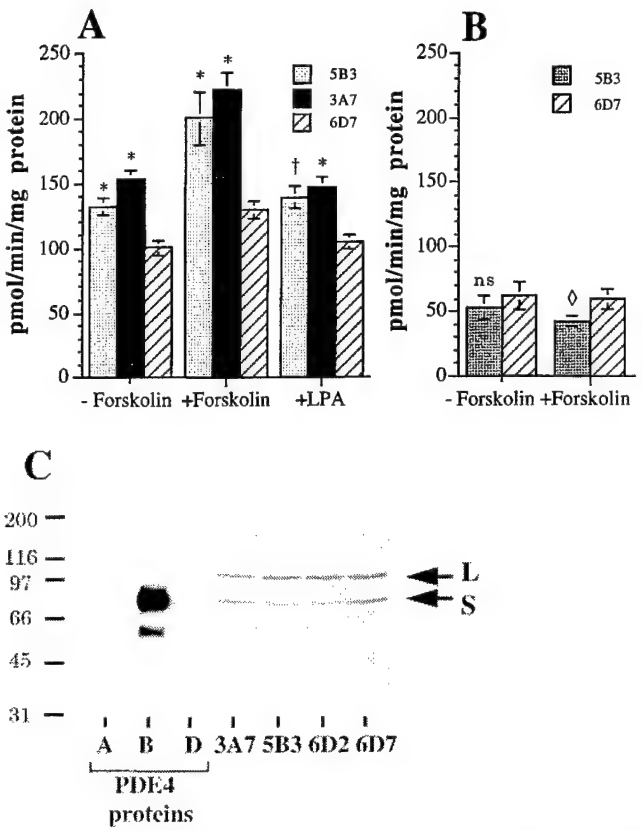

Figure 6. Assay of cAMP-specific PDE activity. (*A*) MDA/β4 (*3A7* and *5B3*) or mock transfectants (*6D7*) plated on collagen I were treated with 50 μM forskolin or 100 nM LPA as noted. Cells were harvested and the cytosolic fraction was assayed for PDE activity as described in Materials and Methods. The PDE activity of the MDA/β4 transfectants was compared with the MDA/mock transfectants for statistical significance: *, $P < 0.002$; †, $P < 0.01$. (*B*) Extracts from cells treated as in *A* were incubated with 100 μM rolipram before assaying for PDE activity to determine how much of the activity in *A* constitutes cAMP-specific PDE (*PDE 4*). Data shown are mean ± standard error of four separate determinations (*A* and *B*). *ns*, not significant; ◇, $P = 0.02$. (*C*) Relative expression of PDE 4B in the MDA-MB-435 transfectants. Extracts (40 μg protein) obtained from the MDA/β4 (*3A7* and *5B3*) and mock (*6D2* and *6D7*) transfectants, as well as purified PDE 4 proteins (short form of variants *A*, *B*, and *D*; 10 ng each; provided by M. Conti) were resolved by SDS-PAGE and immunoblotted with a PDE 4B-specific Ab. *Arrows*, long and short forms of PDE 4B.

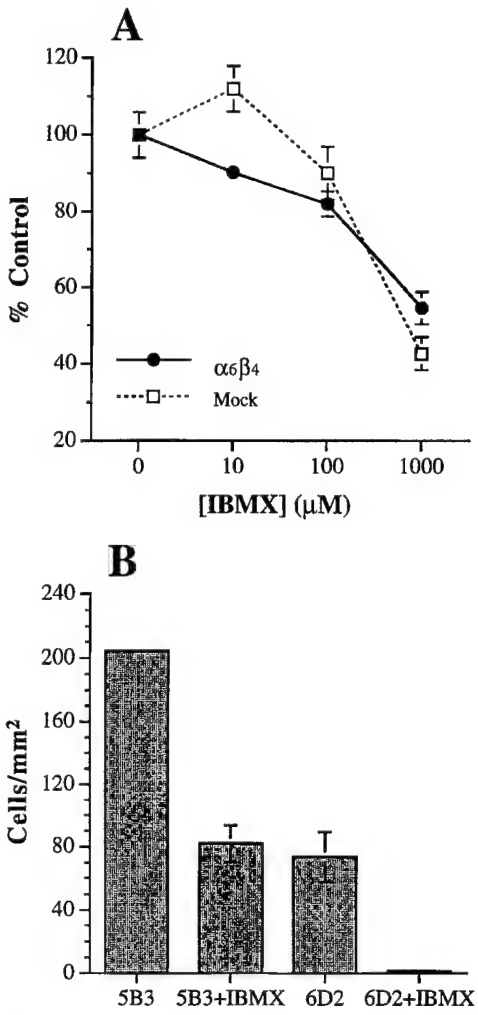

Figure 7. cAMP specific-PDE activity is required for the chemotactic migration and invasion of MDA-MB-435 cells. The MDA/β4 (*5B3*; *squares*) or mock transfectants (*6D7*; *circles*) were treated with varying concentrations (*A*) or 1 mM (*B*) IBMX for 30 min before their use in either an LPA chemotaxis assay (*A*) or a Matrigel chemoinvasion assay (*B*). Data shown represent mean values ± standard deviation of triplicate determinations.

tor, rolipram (data not shown). We also examined the involvement of PDE in carcinoma invasion by treating cells with IBMX before their use in a standard Matrigel invasion assay. A substantial inhibition of invasion was observed in the presence of IBMX in comparison to the solvent control (Fig. 7 *B*).

The necessity of cAMP-specific PDE activity in the formation of lamellae was also assessed. IBMX had no effect on the morphology of the MDA/β4 transfectants in the absence of LPA (Fig. 8, compare *A* with *B*). However, IBMX-treated cells were unable to form the large, ruffling lamellae in response to LPA stimulation in comparison to untreated cells (Fig. 8, compare *C* with *D*). Quantitative analysis of these cell populations revealed that inhibition of PDE activity resulted in an approximate fourfold reduction in the lamellar area of LPA-stimulated MDA/β4 transfectants (Fig. 8 *E*).

Recently, we reported that α6β4 is necessary for the formation and stabilization of lamellae in clone A colon carcinoma cells plated on laminin-1 (32). If PDE activity is needed for lamellae formation as indicated by the above results, IBMX should inhibit the formation of lamellae in clone A cells. To test this possibility, clone A cells were treated with IBMX or a solvent control and then plated onto laminin-1 for 45 min. The control cells formed large fan-shaped lamellae enriched in F-actin when plated on laminin-1 (Fig. 9, *A* and *B*) as we reported previously (32). In contrast, IBMX-treated cells formed small, immature lamellae with a marked reduction in F-actin content (Fig. 9, *C* and *D*). Quantitative analysis of these images re-

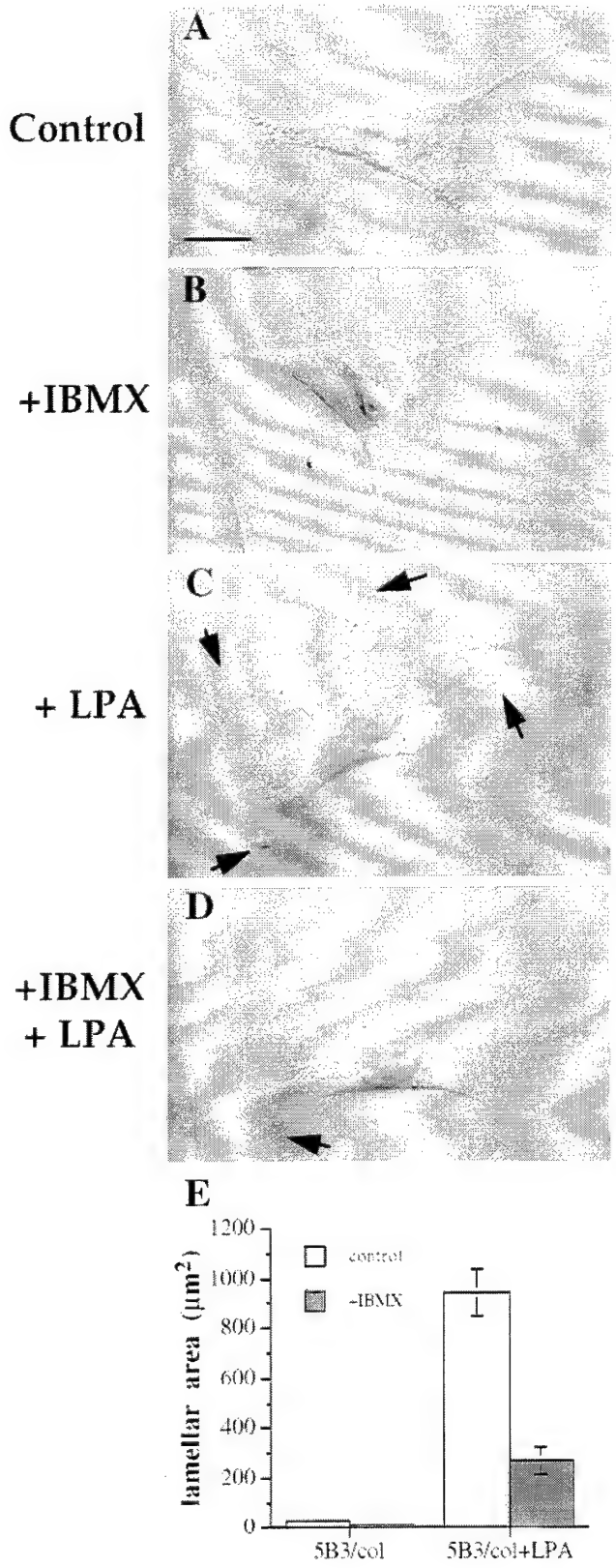

Figure 8. cAMP specific-PDE activity is required for LPA-dependent formation of lamellae in the MDA/β4 transfectants. The MDA/β4 transfectants (*5B3*) were plated on collagen I–coated coverslips. After 2 h, the cells were either left untreated (*A* and *C*) or treated with 1mM IBMX (*B* and *D*) for 30 min. Subsequently, the cells were either left untreated (*A* and *B*) or treated with 100 nM LPA for 5 min (*C* and *D*). The cells were then fixed and visualized using Nomarski DIC optics. (*E*) The effect of LPA and IBMX on lamellar area was quantified using IPLab Spectrum imaging software. Bars represent mean lamellar area ± standard error in which $n > 20$. Of note, IBMX inhibited the LPA-dependent formation of lamellae by 70%.

vealed that IBMX reduced the total lamellar area of clone A cells on laminin-1 by ~75% (629 ± 74 μm^2 for control versus 164 ± 24μm^2 with IBMX). Interestingly, inhibition of PDE activity had no effect on the attachment or spreading of clone A cells on laminin-1 (Fig. 9).

cAMP Metabolism and PI3-K Signaling Are Not Directly Linked in MDA-MB-435 Cells

A possible relationship between cAMP metabolism and PI3-K signaling is of interest given our recent finding that α6β4 stimulates the preferential activation of PI3-K and that this activity is required for invasion and the formation of lamellae (40). To determine if PI3-K activity is required for the cAMP-specific PDE activity we observed in the MDA/β4 transfectants, these cells were incubated in the presence of wortmannin, a specific inhibitor of PI3-K, before extraction and assay of PDE activity. As shown in Fig. 10 *A*, wortmannin had no effect on PDE activity in these cells and it did not inhibit the marked induction of PDE activity that we had observed in response to forskolin stimulation. The possibility also existed that cAMP influences the α6β4-mediated activation of PI3-K. To address this issue, we used the α6-specific mAb G0H3 to cluster α6 integrins on the MDA/β4 transfectants in the presence of the PDE inhibitor IBMX and forskolin. As shown in Fig. 10 *B*, mAb-mediated clustering of α6β4 in MDA/β4 transfectants activated PI3-K markedly compared with cells maintained in suspension, in agreement with our previous results (40). However, treatment of MDA/β4 transfectants with either IBMX or forskolin did not inhibit α6β4-mediated activation of PI3-K (Fig. 10 *B*). In fact, no inhibition of PI3-K was observed when both of these inhibitors were used in combination, a treatment that increases the $[cAMP]_i$ from 4 to 120 pmoles per 10^6 cells (Fig. 5).

Discussion

Recently, we established that the α6β4 integrin promotes carcinoma invasion (3, 32, 40). In the current study, we extend this observation by demonstrating that a major function of α6β4 is to stimulate the chemotactic migration of carcinoma cells, a function that is essential for invasion. This function is consistent with our previous finding that α6β4 is involved in the formation and stabilization of lamellae and filopodia (32). Importantly, the data presented here also provide evidence that α6β4 stimulates chemotaxis and lamellae formation by regulating the $[cAMP]_i$ by a mechanism that involves activation of a rolipram-sensitive, cAMP-specific PDE. Our finding that elevated $[cAMP]_i$ inhibits the formation of lamellae, chemotactic migration, and invasion is in agreement with recent studies indicating that cAMP can function to inhibit or "gate" specific signaling pathways (14, 16, 23). Further-

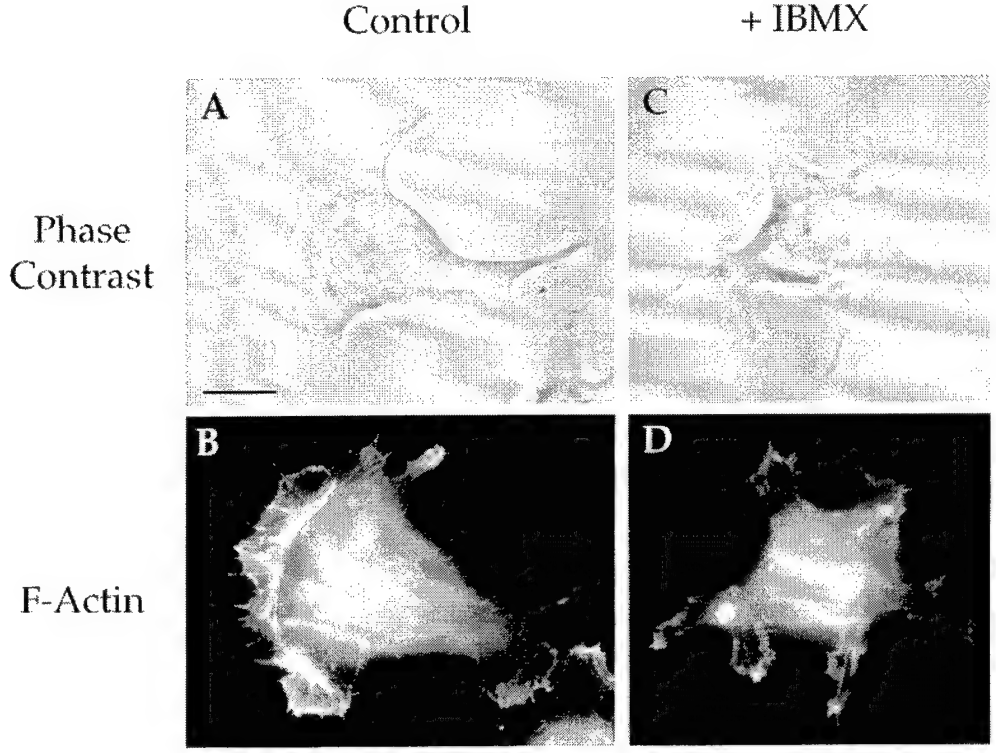

Figure 9. Lamellae formation in clone A colon carcinoma cells requires PDE activity. Clone A colon carcinoma cells were either treated with solvent alone (*A* and *B*) or 1 mM IBMX in solvent (*C* and *D*) and then plated on laminin-1–coated coverslips. After 45 min the cells were fixed and stained for F-actin using TRITC-phalloidin. (*A* and *C*) Phase–contrast images; (*B* and *D*) fluorescence images. Bar, 10 μm.

more, we show that the cAMP-mediated gate does not influence haptotaxis thus providing additional evidence that the signaling events governing chemotaxis and haptotaxis differ (1, 20). Collectively, our results strengthen the hypothesis that α6β4 promotes carcinoma invasion through its ability to regulate signaling pathways required for migration. They also indicate that cAMP metabolism is likely to be an important factor in the regulation of carcinoma invasion and progression.

Although integrins can regulate a number of signaling pathways (4), their ability to influence cAMP metabolism has not been studied extensively. An earlier study, however, did provide evidence that the simultaneous engagement of β2 integrins and tumor necrosis factor (TNF) receptors decreases the $[cAMP]_i$ in neutrophils (30). Interestingly, the reduction in $[cAMP]_i$ observed in response to β2 integrin and TNF receptor engagement in neutrophils is similar to the level of $[cAMP]_i$ suppression that we observed in response to α6β4 expression (~30%). This level of suppression of the total $[cAMP]_i$ is quite impressive given that localized gradients of $[cAMP]_i$ are probably required to facilitate chemotactic migration, as well as for other cell functions that are gated by cAMP. For example, localized gradients of cAMP have been implicated in regulating the direction of growth cone turning (41). It is also important to note that we observed an inverse correlation between the $[cAMP]_i$ and the rate of chemotaxis (compare values in Fig. 4 *A* with Fig. 5 *B*). This observation reinforces the functional significance of α6β4 suppression of the $[cAMP]_i$.

A novel aspect of our study is the finding that integrins, and α6β4 in particular, can regulate the activity of a rolipram-sensitive, cAMP-specific PDE. This family of PDEs is defined as type IV PDE (PDE 4) and consists of a number of structural variants (6). Because all of these variants hydrolyze cAMP with a K_m comparable to the $[cAMP]_i$, it is thought that tissue-specific expression and the state of activity of these variants are the major determinants of their responsiveness to extracellular stimuli (6). Indeed, the major focus of work in this area has been hormonal regulation of PDE activity. Regulation of PDE activity can occur rapidly in response to hormone stimulation through a mechanism that involves PKA-dependent phosphorylation of the enzyme (36, 37). In addition, long-term, hormonal stimulation can actually increase *de novo* synthesis of the cAMP-specific PDEs (6, 43). The data we obtained suggest that expression of α6β4 does not increase the expression of PDE 4B, a predominant PDE variant expressed by MDA-MB-435 cells. For this reason, regulation of PDE 4 activity by α6β4 expression may occur through a mechanism that involves PDE phosphorylation. Another possibility that has been proposed recently is that the subcellular localization of the cAMP-specific PDEs influences their function and activation (17). The possibility that α6β4 increases the association of PDE 4 with either the plasma membrane or cytoskeleton is certainly attractive and could account, at least in part, for its ability to influence cAMP metabolism. Interestingly, LPA stimulation by itself had no effect on either PDE activity or the $[cAMP]_i$ in MDA-MB-435 cells. This observation reinforces our hypothesis that a major function of α6β4 is to release cAMP gating of LPA-stimulated chemotaxis.

In previous studies, we established that an important function of α6β4 in invasive carcinoma cells is its ability to stimulate the formation of lamellae (32). This function of α6β4 is highlighted by the observation in the present study

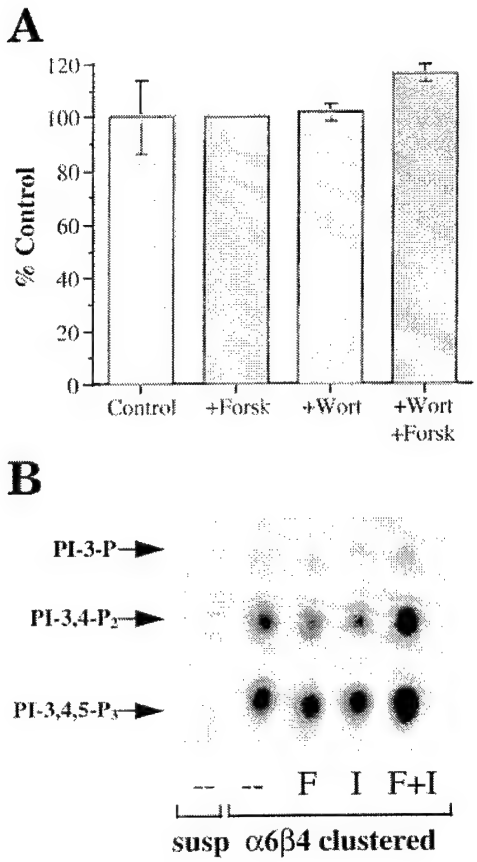

Figure 10. (*A*) Evaluation of PI3-K involvement in PDE activity. The MDA/β4 transfectants were incubated for 30 min in the presence of either forskolin or wortmannin, or both in combination, before assay of PDE activity as described in Materials and Methods. (*B*) Evaluation of the cAMP regulation of PI3-K activity. The MDA/β4 and mock transfectants were incubated in suspension with either forskolin or IBMX or both for 10 min. Subsequently, these cells were either maintained in suspension or incubated with a β4 integrin-specific antibody and allowed to adhere to anti-mouse IgG-coated plates or laminin-1–coated plates for 30 min. Aliquots of cell extracts that contained equivalent amounts of protein were incubated with the anti-phosphotyrosine mAb 4G10 and protein A–Sepharose for 3 h. After washing, the beads were resuspended in kinase buffer and incubated for 10 min at room temperature. The phosphorylated lipids were resolved by thin layer chromatography. *Arrows*, position of the D3-phosphoinositides.

that LPA was able to induce significant lamellae formation only in MDA-MB-435 cells that expressed α6β4 (Fig. 3). Importantly, our finding that PDE activity is necessary for lamellae formation promoted by α6β4 expression implies that a localized suppression of the $[cAMP]_i$ plays an important role in controlling the signaling and cytoskeletal events that are required for lamellae formation. This hypothesis agrees with studies that have shown an inhibitory effect of cAMP on the organization of the actin cytoskeleton (11, 13, 21, 22). Moreover, the formation of lamellae is a dynamic process that is linked to the mechanism of cell migration. Therefore, it is likely that temporal fluxes in the $[cAMP]_i$ regulated by α6β4 contribute to the chemotactic migration of carcinoma cells.

Of particular relevance to our work, Butcher and colleagues (23) reported that cAMP is a negative regulator of leukocyte migration signaled through the classical chemoattractants. In this model, cAMP impedes or gates RhoA-mediated leukocyte integrin activation and adhesion. Our results support their conclusion that cAMP inhibits chemotactic migration. Importantly, we also provide evidence for an integrin-mediated mechanism of regulating the $[cAMP]_i$ to facilitate migration. Our findings support the work of the Butcher group because LPA is a potent activator of Rho (29) and, in fact, we have observed that the expression of a dominant-negative Rho can inhibit both LPA-stimulated chemotactic migration and invasion of MDA-MB-435 cells (O'Connor, K.L., and A.M. Mercurio, unpublished observation). Thus, a possibility worth investigating is that LPA-mediated activation of Rho is gated by cAMP and that α6β4 releases cAMP gating by increasing cAMP-specific PDE activity and thereby enhances Rho activation. A likely target of Rho activation is the actin cytoskeleton (44), which is consistent with the reported effects of cAMP on the cytoskeleton (11, 13, 21, 22), as well as our demonstration that cAMP inhibits lamellae formation. Although Rho has been linked to stress fiber formation and not lamellae formation in fibroblasts (25), much less is known about Rho function in epithelial-derived cells. In fact, our preliminary data suggest that LPA-induction of lamellae formation in the MDA/β4 transfectants is inhibited by expression of a dominant-negative Rho. Other integrins, especially the β1 integrins that mediate the adhesive interactions required for chemotactic migration, are another potential target of Rho (25, 34). It is worth noting in this context that expression of α6β4 has been shown to alter the function of collagen I–binding integrins in breast carcinoma cells (42).

The ability of α6β4 to promote lamellae formation and carcinoma invasion is dependent upon its preferential activation of a PI3-K and Rac signaling pathway (40). Our current finding that the release of cAMP gating by α6β4 is also required for these events raised the issue of a possible link between cAMP and the PI3-K/Rac pathway. Such a link was suggested, for example, by the finding that the interleukin-2 dependent activation of PI3-K is inhibited by cAMP (28). In our experiments, however, pharmacological stimulation of cAMP levels had no effect on the ability of α6β4 to activate PI3-K even under conditions in which the $[cAMP]_i$ increased 30-fold over basal levels. Our data also indicate that PI3-K probably does not function upstream of cAMP-specific PDE because wortmannin did not inhibit the activity of this enzyme. We conclude from these findings that PI3-K and cAMP-specific phosphodiesterase function in tandem to promote lamellae formation and chemotactic migration but they are components of distinct signaling pathways.

An interesting finding in the present study is that the ability of α6β4 to stimulate chemotactic migration and suppress the $[cAMP]_i$ can be independent of the adhesive function of this integrin. Although the laminins are the only known matrix ligands for α6β4 (26), expression of this integrin also stimulated chemotactic migration on a collagen I matrix and this migration was not inhibited by an α6-function blocking mAb. The possibility that the effects of α6β4 expression on migration result from a de-

crease of α6β1 expression is discounted by the fact that expression of the α6β4 ΔCYT integrin had no effect on either chemotaxis, cAMP levels or PDE activity even though expression of this mutant integrin eliminates α6β1 expression in these cells (38). The observation that the ability of α6β4 to promote chemotactic migration can be independent of its adhesive function is in agreement with several recent studies by our group and others that have revealed 'ligand-independent' functions for the α6 integrins in carcinoma cells (3, 5, 8). Insight into the possible mechanism of this phenomenon was provided by a recent study that demonstrated self-association of the β4 cytoplasmic domains, a process that could initiate intracellular signaling events independently of ligand binding (33). One important implication of these findings is that the ability of α6β4 to influence cAMP metabolism and stimulate the chemotactic migration of carcinoma cells need not be limited to sites of contact with laminin-containing matrices. This possibility is supported by the numerous studies that have implicated α6β4 as a major determinant of carcinoma invasion and progression (10, 35, 47).

In summary, we have demonstrated that the α6β4 integrin can stimulate lamellae formation and chemotactic migration of invasive carcinoma cells by increasing the activity of a rolipram-sensitive cAMP specific-PDE and lowering the $[cAMP]_i$. This cAMP specific-PDE functions in tandem with a PI3-K/Rac pathway, that is also regulated by α6β4, and is required for carcinoma invasion and lamellae formation. The essence of our findings is that the α6β4 integrin stimulates the chemotactic migration of carcinoma cells through its ability to influence key signaling events that underlie this critical component of carcinoma invasion.

We would also like to thank M. Conti and S. Iona (Stanford University, Stanford, CA) for their generous contribution of the PDE 4 antibodies, as well as S. Akiyama for the β1 integrin antibody. We also thank R. Falcioni, (Regina Elena Cancer Institute, Rome, Italy), R. Bachelder, I. Rabinovitz, and D. Senger (all three from Beth Israel Deaconess Medical Center, Boston, MA) for their comments and discussions regarding the manuscript.

This work was supported by grants from the United States Army Medical Research and Material Command to K. O'Connor (DAMD17-98-1-8033), L.M. Shaw (DAMD17-97-1-7313), and A.M. Mercurio (DAMD17-96-1-6199) and a grant from the National Institutes of Health to A.M. Mercurio (CA-44704).

Received for publication 24 September 1998 and in revised form 27 October 1998.

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Brief Report

RhoA Function in Lamellae Formation and Migration Is Regulated by the α6β4 Integrin and cAMP Metabolism

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Abstract. Clone A colon carcinoma cells develop fan-shaped lamellae and exhibit random migration when plated on laminin, processes that depend on the ligation of the α6β4 integrin. Here, we report that expression of a dominant negative RhoA (N19RhoA) in clone A cells inhibited α6β4-dependent membrane ruffling, lamellae formation, and migration. In contrast, expression of a dominant negative Rac (N17Rac1) had no effect on these processes. Using the Rhotekin binding assay to assess RhoA activation, we observed that engagement of α6β4 by either antibody-mediated clustering or laminin attachment resulted in a two- to threefold increase in RhoA activation, compared with cells maintained in suspension or plated on collagen. Antibody-mediated clustering of β1 integrins, however, actually suppressed Rho A activation. The α6β4-mediated interaction of clone A cells with laminin promoted the translocation of RhoA from the cytosol to membrane ruffles at the edges of lamellae and promoted its colocalization with β1 integrins, as assessed by immunofluorescence microscopy. In addition, RhoA translocation was blocked by inhibiting phosphodiesterase activity and enhanced by inhibiting the activity of cAMP-dependent protein kinase. Together, these results establish a specific integrin-mediated pathway of RhoA activation that is regulated by cAMP and that functions in lamellae formation and migration.

Key words: carcinoma • protein kinase A • G-protein • phosphodiesterase • cytoskeleton

Introduction

The organization and remodeling of the actin cytoskeleton are controlled by the Rho family of small GTPases, which includes Rho, Rac, and cdc42. These proteins have been implicated in the formation of stress fibers, lamellipodia, and filopodia, respectively (reviewed in Hall, 1998). Although much of our knowledge on their function has been obtained from studies with fibroblasts, other activities for these Rho GTPases have been observed recently in cells of epithelial origin. For example, Rac and cdc42 are required to maintain apical–basal polarity in epithelia (Jou and Nelson, 1998). Rho, interestingly, has been implicated in membrane ruffling in epithelial cells (Nishiyama et al., 1994; Fukata et al., 1999), a process attributed to Rac in fibroblasts (Hall, 1998). These findings are of particular interest with respect to our understanding of epithelial cell migration. The migration and invasion of epithelial-derived carcinoma cells are important phenomena that require the involvement of Rho GTPases (Keely et al., 1997; Shaw et al., 1997; Yoshioka et al., 1998; Itoh et al., 1999). For these reasons, it is essential to define the factors that regulate the function of Rho GTPases in carcinoma cells and to characterize the mechanisms by which they contribute to the dynamics of migration. For example, although cell adhesion has been reported to activate RhoA (Barry et al., 1997; Ren et al., 1999), little is known about the involvement of specific integrins in adhesion-dependent RhoA activation or in the regulation of RhoA-dependent functions.

Recent studies by our group have highlighted a pivotal role for the integrin α6β4 in the migration and invasion of carcinoma cells, as well as in epithelial wound healing (Lotz et al., 1997; Rabinovitz and Mercurio, 1997; Shaw et al., 1997; O'Connor et al., 1998; Rabinovitz et al., 1999). Although it is well established that α6β4 functions in the formation and stabilization of hemidesmosomes (Borradori and Sonnenberg, 1996; Green and Jones, 1996), our findings revealed a novel role for this integrin in the formation of actin-rich cell protrusions at the leading edges of carcinoma cells and in the migration of these cells

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(Rabinovitz and Mercurio, 1997; Rabinovitz et al., 1999). Moreover, we demonstrated the importance of cAMP metabolism in these events (O'Connor et al., 1998). Given the recent interest in the participation of RhoA in migration, we examined the hypothesis that RhoA is essential for the formation of actin-rich cell protrusions, the migration of carcinoma cells and, more importantly, that the activity of RhoA is regulated by the α6β4 integrin. In addition, we assessed the involvement of cAMP metabolism in these events.

Materials and Methods

Cells and Antibodies

Clone A cells, originally isolated from a poorly differentiated colon adenocarcinoma (Dexter et al., 1979), were used in all experiments. For each experiment, adherent cells were harvested by trypsinization, rinsed three times with RPMI medium containing 250 μg/ml heat-inactivated BSA (RPMI/BSA), and resuspended in RPMI/BSA. Where indicated, cells were treated with 1 mM isobutylmethylxanthine (IBMX)[1] or 15 mM H-89 (Calibiochem-Novabiochem, Int.) for 15 min before use. The following antibodies were used in this study: MC13, mouse anti-β1 integrin mAb (obtained from Steve Akiyama, National Institutes of Health, Research Triangle Park, NC); K20, mouse anti-β1 integrin mAb (Immunotech); 439-9B, rat anti-β4 integrin mAb (obtained from Rita Falcioni, Regina Elena Cancer Institute, Rome, Italy); mouse anti-HA mAb (Roche Biochemicals); rabbit anti-RhoA polyclonal antibody (Santa Cruz Biotechnology); and anti-Rac1 (Transduction Laboratories).

To obtain expression of N19RhoA and N17Rac1, adherent cells were harvested using trypsin, rinsed with PBS, and suspended in electroporation buffer (20 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4-$7H_2O$, 5 mM glucose). Cells were cotransfected with 1 mg of either pCS2-(n)β-gal or pGFP (green fluorescent protein) and 4 μg of either control vector or vector containing HA-tagged N19RhoA (provided from Alex Toker, Beth Israel Deaconess Medical Center, Boston, MA) or GST-tagged N17Rac1 (obtained from Margaret Chou, University of Pennsylvania) by electroporation at 250V and 500 μFd. Subsequently, cells were plated in complete growth medium containing 0.05% sodium butyrate and used for experiments 48 h after the initial transfection. Expression of the recombinant proteins was confirmed by concentrating extracts of transfected cells with an HA-specific mAb or glutathione-coupled beads and subsequent immunoblotting for RhoA or Rac1, respectively.

Microscopic Analyses

Glass coverslips were coated overnight at 4°C with collagen I (50 μg/ml; Collagen Corp.) or laminin-1 purified from EHS tumor (20 μg/ml; provided by Hinda Kleinman, NIDR, Bethesda, MD) and then blocked with BSA (0.25% in RPMI). Cells were plated on these coverslips for 30–40 min, rinsed with PBS, fixed, and then permeabilized as described previously (O'Connor et al., 1998). For immunofluorescence, cells were incubated with 1 μg/ml of K20 (anti-β1) and anti-RhoA antibody diluted in block solution (3% BSA/1% normal donkey serum in PBS) for 30 min, rinsed four times with PBS, and then incubated for 30 min with a 1:400 dilution of anti-mouse IgG Cy2- and anti-rabbit IgG Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Images of cells were captured digitally, analyzed, and lamellar area quantified as described previously (Rabinovitz and Mercurio, 1997; O'Connor et al., 1998).

Migration Assays

The lower compartments of Transwell chambers (6.5-mm diam, 8-μm pore size; Costar) were coated for 30 min with 15 μg/ml laminin-1 diluted in RPMI medium. RPMI/BSA was added to the lower chamber and cells (1×10^5) suspended in RPMI/BSA were added to the upper chamber. After incubating for 5 h at 37°C, cells were removed from the upper chamber with a cotton swab and cells that had migrated to the lower surface of the membrane were fixed, stained with crystal violet or for β-galactosidase (β-gal), and quantified as described previously (Shaw et al., 1997).

RhoA Activity

RhoA activity was assessed using the Rho-binding domain of Rhotekin as described (Ren et al., 1999). In brief, cells (3×10^6) were plated onto 60-mm dishes coated with LN-1 (20 μg/ml) or collagen I (50 μg/ml) for 30 min and extracted with RIPA buffer (50 mM Tris, pH 7.2, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS, 10 mM $MgCl_2$, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 4 μg/ml aprotinin, and 2 mM PMSF). Alternatively, cells were incubated with 8 μg of anti-β1 mAb mc13 or anti-β4 rat mAb 439-9B for 30 min, rinsed, plated on 60-mm dishes coated with 50 μg of either anti-mouse or anti-rat IgG, respectively, for 30 min, and then extracted. After centrifugation at 14,000 *g* for 3 min, the extracts were incubated for 45 min at 4°C with glutathione beads (Pharmacia Biotech) coupled with bacterially expressed GST–RBD (Rho-binding domain of Rhotekin) fusion protein (provided by Martin Schwartz, Scripps Research Institute, La Jolla, CA), and then washed three times with Tris buffer, pH 7.2, containing 1% Triton X-100, 150 mM NaCl, and 10 mM $MgCl_2$. The RhoA content in these samples was determined by immunoblotting samples using rabbit anti-RhoA antibody.

Results

Clone A colon carcinoma cells develop fan-shaped lamellae and exhibit random migration when plated on laminin-1, processes that are dependent on both the α6β4 and β1 integrins. In contrast, the β1 integrin-mediated adhesion and spreading of these cells on collagen I does not induce significant lamellae formation or migration (Rabinovitz and Mercurio, 1997; Shaw et al., 1997). To examine the hypothesis that RhoA functions in α6β4-dependent lamellae formation, clone A cells were cotransfected with a GFP construct and either a dominant negative RhoA (N19RhoA) or a control vector. Subsequently, the cells were plated onto laminin-1 and examined by phase-contrast microscopy. Clone A cells that expressed the control vector developed large lamellae with ruffled edges (Fig. 1 A). In contrast, cells that expressed N19RhoA developed only a few small, fragmented lamellae that were devoid of membrane ruffles (Fig. 1 B). Quantitative analysis of these images revealed that expression of N19RhoA reduced lamellar area by 80% in comparison to cells that expressed the control vector (Fig. 1 D). Interestingly, expression of a GST-tagged, dominant negative Rac1 (N17Rac1) did not inhibit either lamellae formation or membrane ruffling in clone A cells (Fig. 1, C and D), although this construct has been shown to inhibit p70 S6 kinase (Chou and Blenis, 1996) and invasion (Shaw et al., 1997).

Expression of N19RhoA inhibited the migration of clone A cells on laminin-1 by 70% (Fig. 2 A). In contrast, expression of N17Rac did not inhibit the migration of clone A cells (Fig. 2 A), although it did inhibit the migration of 3T3 cells by 85% (data not shown). Importantly, expression of N19RhoA had only a modest effect on cell spreading because cells expressing N19RhoA plated on collagen–I spread to ~80% of the surface area occupied by control cells (Fig. 1 E). Expression of N19RhoA and N17Rac1 in clone A cells was confirmed by immunoblotting (Fig. 1, F and G).

Our observation that RhoA functions in lamellae for-

[1]*Abbreviations used in this paper:* β-gal, β-galactosidase; GFP, green fluorescent protein; IBMX, isobutylmethylxanthine; LPA, lysophosphatidic acid; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; RBD, Rho-binding domain of Rhotekin.

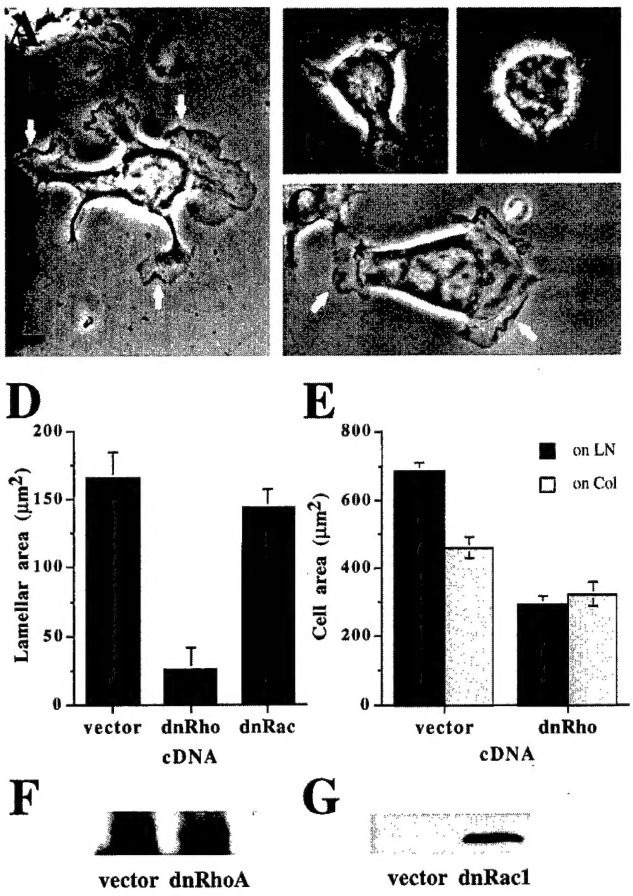

Figure 1. Dominant negative RhoA inhibits membrane ruffling and lamellae formation in clone A cells in response to laminin-1. Clone A cells were cotransfected with a GFP construct and either a control vector or a vector encoding N19RhoA or N17Rac as described in Materials and Methods. Cells were plated onto laminin-coated coverslips for 40 min, fixed, and assessed by phase-contrast microscopy. A–C, Phase-contrast microscopy of vector control (A), N19RhoA (B, two panels), or N17Rac (C) transfected cells. Note large lamellae and membrane ruffles in control and N17Rac transfected cells (open arrow in A and C), but not in cells that express N19RhoA (B). Representative GFP-positive cells are shown. D, Quantitative analysis of the lamellar area of transfected, GFP-positive cells was obtained by digital imaging. Lamellae are defined as broad, flat cellular protrusions rich in F-actin and devoid of membrane-bound vesicles. E, Quantitative analysis of total area covered by cells transfected with either vector control or N19RhoA when plated on laminin-1 (dark bars) or collagen I (light bars). Bars represent mean area ± SEM in which $n > 20$ (D, E). F and G, Transfected cells were extracted with RIPA buffer and either immunoprecipitated with HA-specific mAb and immunoblotted for RhoA (F), or concentrated using glutathione-Sepharose and immunoblotted for Rac1 (G). Representative blots are shown.

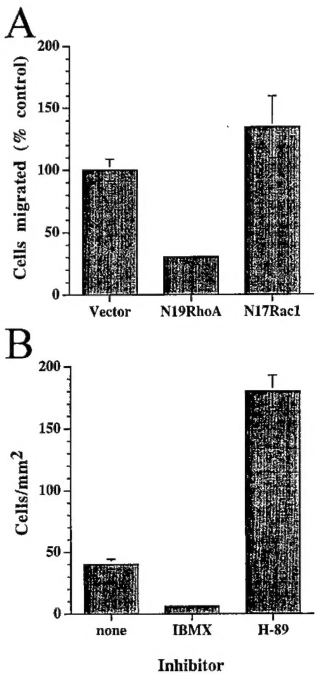

Figure 2. Effects of dominant negative RhoA (N19RhoA) and cAMP metabolism on laminin-1 stimulated migration. A, Clone A cells that had been cotransfected with a β-gal cDNA and either N19RhoA, N17Rac1, or control vector were assayed for migration on laminin-1 as described in Materials and Methods. Migration was scored as the relative number of β-gal staining cells migrated compared with the vector only control. Transfection rates were comparable. B, Clone A cells were left untreated or treated with 1 mM IBMX or 15 μM H-89 for 15 minutes and then assayed for laminin-1 mediated migration as described in Materials and Methods. Migration rates were reported as the number of cells migrated per mm^2. Bars represent mean ± SD from triplicate determinations.

mation and the migration of clone A cells, in conjunction with our previous finding that these events require the engagement of the α6β4 integrin (Rabinovitz and Mercurio, 1997), indicated that α6β4 may mediate the activation of RhoA. To assess RhoA activation, we used the RBD to capture GTP-bound RhoA from cell extracts (Ren et al., 1999). As shown in Fig. 3, the interaction of clone A cells with laminin-1, which requires α6β4, resulted in a significant amount of RhoA retained by RBD in comparison to the interaction of these cells with collagen I, which does not involve α6β4 directly. These experiments were performed with cells that had been attached to laminin for 30 min because membrane ruffling was most apparent at this time. Quantitative analysis of the results obtained in four independent experiments revealed a threefold greater increase in RhoA activation in cells plated on laminin-1 than in cells plated on collagen (Fig. 3 B). To establish the ability of α6β4 to activate RhoA more definitively, we used integrin-specific mAbs to cluster both α6β4 and β1 integrins. As shown in Fig. 3, C and D, clustering of α6β4 resulted in an approximate two- to threefold higher level of RhoA activity in comparison to cells maintained in suspension. Interestingly, clustering of β1 integrins actually decreased RhoA activation in comparison to cells maintained in suspension (Fig. 3), even though clone A cells express similar surface levels of both integrins (Lee et al., 1992). Similar results were obtained between 5 and 30 min of antibody clustering (data not shown).

The involvement of cAMP metabolism in migration, lamellae formation, and α6β4-mediated RhoA activation was investigated using both IBMX, a phosphodiesterase (PDE) inhibitor, and H-89, a cAMP-dependent protein kinase (PKA) inhibitor. IBMX treatment, which prevents cAMP breakdown, inhibited the migration of clone A cells on laminin-1 almost completely (Fig. 2 B). In contrast, inhibition of PKA with H-89 increased the rate of migration by fourfold (Fig. 2 B). Together, these data indicate that cAMP inhibits or "gates" carcinoma migration and lamellae formation, in agreement with our previous findings (O'Connor et al., 1998). To establish the involvement of cAMP metabolism in the α6β4-mediated activation of

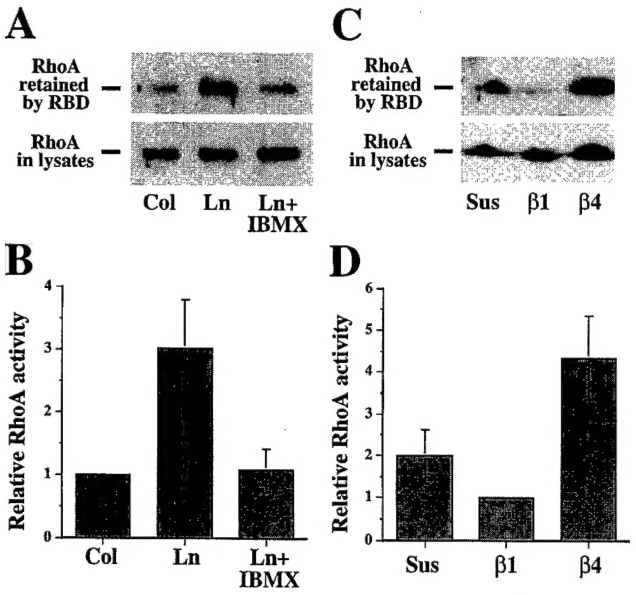

Figure 3. Engagement of the α6β4 integrin by either laminin-1 or antibody-mediated clustering activates RhoA. A and B, Clone A cells were plated on either collagen or laminin for 30 min or pretreated with 1 mM IBMX for 15 min and then plated on laminin for 30 min. Cell extracts were assayed for Rhotekin binding activity as described in Materials and Methods. C and D, Cells were either left in suspension (sus) or clustered with either β1- or β4-specific antibodies for 30 min as described in Materials and Methods. Cell extracts were assessed for RhoA activity by RBD binding. For these experiments, the total RhoA bound to the RBD (top panels in A and C) was normalized to the RhoA content of cell extracts (bottom panels in A and C). A and C, Representative immunoblots from these experiments are shown. B and D, Quantitative analysis of the results obtained by densitometry is provided. Bars represent mean of four separate experiments ± SEM.

RhoA, we used IBMX in the RBD assay. As shown in Fig. 3 A, pretreatment of clone A cells with IBMX before plating on laminin-1 reduced the level of RhoA activation to that observed in cells plated on collagen. Importantly, IBMX did not inhibit either cell adhesion or spreading (Fig. 4, C and E). Similar results were obtained with integrin clustering (data not shown). These observations implicate cAMP metabolism in the α6β4-mediated activation of RhoA.

The data reported here raise the possibility that α6β4 influences RhoA localization because activation of RhoA is thought to involve its translocation to membranes (Bokoch et al., 1994). To address this issue, clone A cells plated on either laminin-1 or collagen I were immunostained with a RhoA-specific antibody, as well as a β1-integrin–specific antibody to mark membranes. In cells plated on collagen I, RhoA immunostaining was confined largely to the cytosol and it was distinct from the β1-integrin staining of the plasma membrane (Fig. 4 A). In contrast, the α6β4-dependent interaction of clone A cells with laminin-1 resulted in the translocation of RhoA to membrane ruffles at the edges of lamellae where it colocalized with β1 integrin staining (Fig. 4 B). However, RhoA did not colocalize with β1 integrins on the plasma membrane along the cell body (Fig. 4 B). To assess the influence of cAMP metabolism on RhoA localization, clone A cells were pretreated with either IBMX or H-89 before plating on laminin-1. Inhibition of PDE activity with IBMX dramatically inhibited membrane ruffling and abolished RhoA localization in the few ruffles that persisted after IBMX treatment (Fig. 4, C and E). Conversely, inhibition of cAMP-dependent PKA with H-89 resulted in an apparent increase in membrane ruffling and RhoA localization in membrane ruffles (Fig. 4, D and F).

Discussion

Recently, we established that the α6β4 integrin stimulates the migration of carcinoma cells and enhances the formation of actin-rich protrusions, including lamellae and membrane ruffles (Shaw et al., 1997; Rabinovitz and Mercurio, 1997; O'Connor et al., 1998; Rabinovitz et al., 1999). In this study, we advance our understanding of the mechanism by which α6β4 functions in these dynamic processes by demonstrating that ligation of α6β4 with either antibody or laminin-1 results in the activation of RhoA and its translocation from the cytosol to membrane ruffles at the leading edges of migrating carcinoma cells. Importantly, we also provide evidence that the α6β4-mediated activation of RhoA is necessary for lamellae formation, membrane ruffling, and migration. Furthermore, we establish that these events are regulated by cAMP metabolism and that they can occur independently of Rac1 involvement.

Our findings strengthen the evidence that integrins can participate in the activation of RhoA. Much of the evidence supporting integrin activation of RhoA had been based largely on the observation that integrin activation leads to the Rho-dependent formation of stress fibers and focal adhesions (Ren et al., 1999; Schoenwaelder and Burridge, 1999). Recently, the development of a biochemical assay for RhoA activation using the ability of GTP-bound RhoA to associate with the Rho-binding domain of Rhotekin has enabled a more rigorous and sensitive assessment of the mechanism of RhoA activation (Ren et al., 1999). Using this assay, cell attachment to fibronectin was shown to activate RhoA and that the level of activation was augmented by serum or lysophosphatidic acid (LPA). In our study, we extend this observation by providing evidence that a specific integrin, α6β4, can activate RhoA, as assessed by both Rhotekin binding and translocation to membrane ruffles. An interesting and unexpected finding obtained in our study is that the α6β4 integrin is a more effective activator of RhoA than β1 integrins in clone A cells. In fact, antibody-mediated ligation of β1 integrins actually suppressed RhoA activation. Because we used carcinoma cells in our study, the potent activation of RhoA we observed in response to α6β4 ligation could have resulted from a cooperation of α6β4 with a secreted growth factor or activated oncogene. However, if cooperative signaling between integrins and such factors occurs in these cells, it is specific for α6β4 because clustering of β1 integrins did not activate RhoA.

Our findings implicate an important role for RhoA in the formation of membrane ruffles and lamellae. Specifically, the expression of N19RhoA in clone A cells attached to laminin resulted in the appearance of frag-

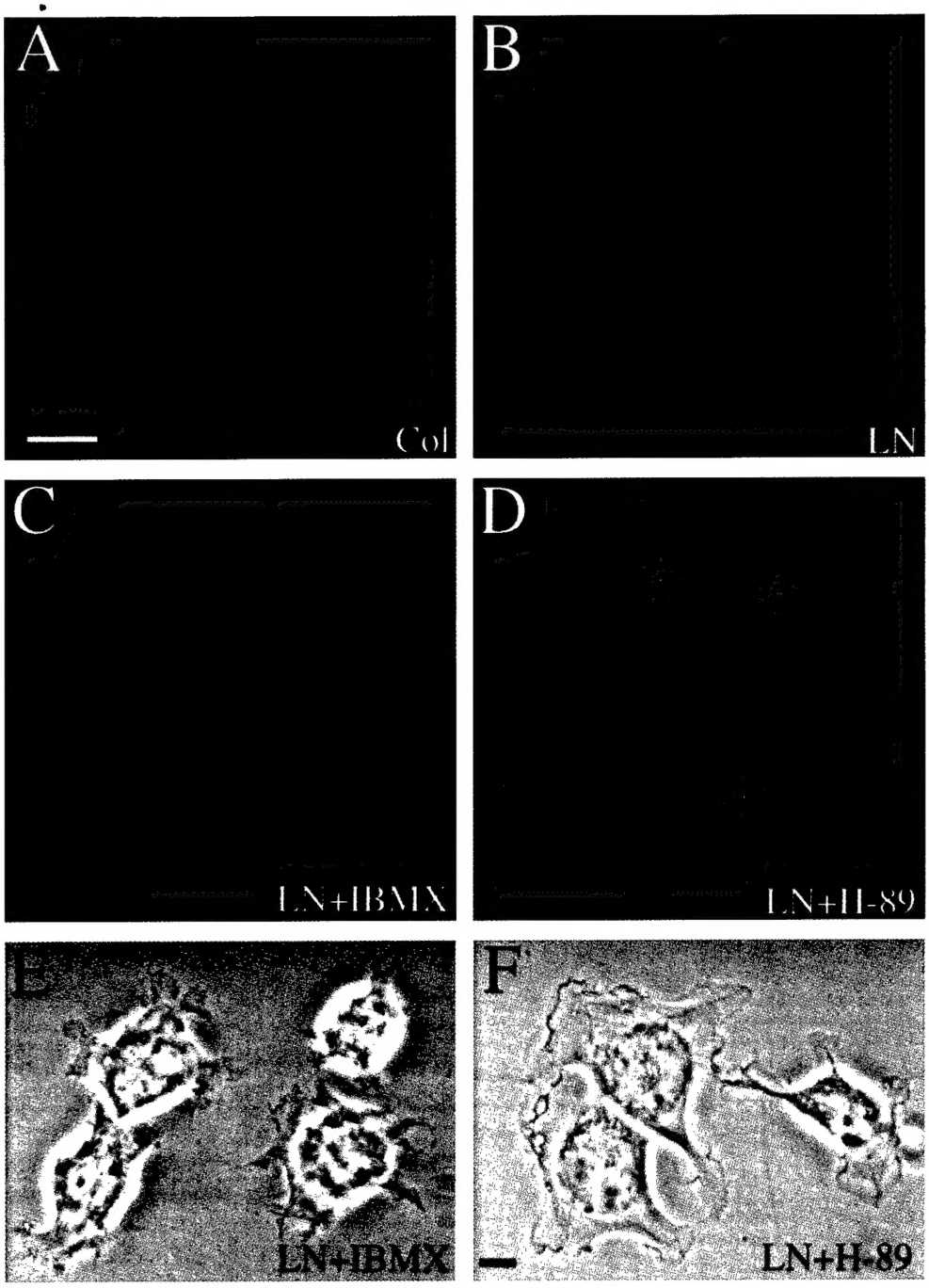

Figure 4. Laminin-1, but not collagen-I, promotes the colocalization of RhoA and β1 integrin in membrane ruffles in a cAMP-sensitive manner. A–D, Clone A cells were plated on either collagen I (A) or laminin-1 (B–D) for 30 min, fixed, and stained for both β1 integrin and RhoA using indirect immunofluorescence as described in Materials and Methods. To assess the impact of cAMP signaling on recruitment of RhoA to the plasma membrane, cells were pretreated with 1 mM IBMX (C) or 15 μM H-89 (D) for 15 min before plating cells on laminin-1. Images were captured digitally ~1 μm from the basal surface using a Bio-Rad confocal microscope. Red color represents RhoA; green, β1 integrin; yellow, β1 and RhoA colocalization. E and F, Phase-contrast micrographs of cells treated with IBMX (E) or H-89 (F) and plated on laminin depict the general impact of cAMP metabolism on membrane ruffling. Bars, 10 μm.

mented, immature lamellae and a loss of membrane ruffles. These results are of interest in light of recent reports that Rho kinase, a downstream effector of Rho, promotes membrane ruffling in epithelial-derived cells (Nishiyama et al., 1994; Fukata et al., 1999) by a mechanism that involves Rho kinase-mediated phosphorylation of adducin (Fukata et al., 1999). Moreover, both Rho and Rho kinase have been implicated in tumor cell invasion (Yoshioka et al., 1998; Itoh et al., 1999). Together, these findings along with our previous work that established the ability of α6β4 to promote carcinoma migration and invasion (Rabinovitz and Mercurio, 1997; Shaw et al., 1997; O'Connor et al., 1998), suggest that α6β4-mediated regulation of the Rho/Rho kinase pathway is an important component of carcinoma progression. It is also possible that the α6β4-mediated activation of RhoA contributes to migration and invasion by activating the adhesive functions of other integrins. RhoA can activate integrin-mediated adhesion in leukocytes (Laudanna et al., 1997) and is believed to participate in adhesion in other cell types (Nobes and Hall, 1999). Our observation that RhoA and β1 integrins colocalize in membrane ruffles in response to α6β4 ligation raises the possibility that RhoA influences the function of β1 integrins, which are essential for migration and invasion.

Interestingly, expression of the dominant negative

N17Rac in clone A cells had no inhibitory effect on either membrane ruffling, lamellae formation, or migration. Although it is well established that Rac functions in lamellipodia formation in fibroblasts (Hall, 1998) and in the migration of several cell types (e.g., see Keely et al., 1997; Shaw et al., 1997; Nobes and Hall, 1999), recent studies have highlighted the complexity of Rac involvement in these dynamic processes. For example, Rac activation can also inhibit migration by promoting cadherin-mediated cell–cell adhesion (Hordijk et al., 1997; Sander et al., 1999) and by downregulating Rho activity (Sander et al., 1999). Nonetheless, Rac activation stimulates membrane ruffling under conditions in which it also promotes cell–cell adhesion (Sander et al., 1999). Clone A cells, therefore, may represent the first example of a cell type in which both membrane ruffling and migration are Rac-independent.

Our results highlight the importance of cAMP metabolism in the activation and localization of RhoA. Our finding that cAMP inhibits RhoA activation and translocation to membrane ruffles is consistent with our previous report that linked the ability of α6β4 to promote carcinoma migration with its ability to alter cAMP metabolism (O'Connor et al., 1998). In addition, these results substantiate other studies that indicated an inhibitory effect of cAMP on RhoA activity (Lang et al., 1996; Laudanna et al., 1997; Dong et al., 1998). The basis for this inhibition may be the direct phosphorylation of RhoA by PKA (Lang et al., 1996). In this context, α6β4 may contribute to RhoA activation by increasing the activity of a cAMP-dependent PDE and subsequently reducing PKA activity, as we have suggested previously (O'Connor et al., 1998). However, the fact that we observed RhoA activation in response to antibody-mediated clustering of α6β4 suggests this integrin can be linked directly to RhoA activation. This direct activation of RhoA would permit α6β4 to augment pathways, such as LPA signaling, that involve RhoA activation. In conclusion, the results reported here establish a specific integrin-mediated pathway of RhoA activation that is regulated by cAMP and functions in lamellae formation and migration.

We thank Steve Akiyama, Rita Falcioni, Hynda Kleinman, Martin Schwartz, and Alex Toker for reagents. We also acknowledge Isaac Rabinovitz and Robin Bachelder for helpful discussions.

This work was supported by the United States Army Medical Research and Materiel Command Grants DAMD17-98-1-8033 (to K.L. O'Connor) and DAMD17-96-1-6199 (to A.M. Mercurio), as well as National Institutes of Health Grant CA80789 (to A.M. Mercurio) and the Harvard Digestive Diseases Center.

Submitted: 18 October 1999
Revised: 17 December 1999
Accepted: 20 December 1999

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